# Neuroprotective Effect of Ultra-High Dose of Methylcobalamin in Models of Mononeuropathy in Rats

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**Shortened Tittle:** Methylcobalamin in mononeuropathy

**Summary**

We have evaluated the neuroprotective actions of Methylcobalamin 500µ/kg (MCA) by using partial sciatic nerve ligation (PSNL) and sciatic nerve crush injury (SNCI) models in wistar rats. The parameters used were thermal hyperalgesia (TH), motor function test (MFT) and motor nerve conduction velocity (MNCV). A steady improvement in TH was seen in MCA treated animals on day $8^{th}$ (9.61±0.19) and day $7^{th}$ (9.41±0.06 s) in the models of PSNL and SNCI respectively. There was a reduction in pain which was observed behaviorally in MFT in both the models and also was observed an improvement in MNCV of MCA treated animals which steadily increased on $15^{th}$ day (38.53±2.69m/s) and $30^{th}$ day(47.63± 2.12m/s), which was equivalent to sham treated group, $30^{th}$ day (47.86 ± 2.59m/s) indicating that MCA has a promising neuroprotective action in PSNL and SNCI in rats.

**Keywords:** Methylcobalamin, Partial sciatic nerve ligation , Sciatic nerve crush injury.

## Introduction

It is now very well known that events like nerve ligation directly causes mechanical injury to the nerve and symptoms produced in such animals resemble the symptoms produced in causalgia in humans. The animal model mimics both hyperalgesia (increased response to noxious stimuli) and allodynia (pain response to low threshold stimulus) **[1,2,3,4]**. Furthermore, it is also reported that extent of nerve trapped in the ligature also affects the effects elicited post surgery and most importantly the pain so produced post operatively is like burning sensation similar to burning sensation in causalgic humans **[3,5,6]**. Direct mechanical injury or ischemia or both can cause acute endothelial injury that can result in endothelial edema, agranulocyte plug or microvascular thrombosis. These factors interrupt the reflow and can cause continuous fiber injury. Moreover, endoneural edema may develop due to microvascular compression. Toxic substances released from neutrophils and macrophages after injury can impair tissue protection in normal conditions and permit the accumulation of free oxygen radicals which increase tissue destruction and cause tissue damage **[5]**. Ultra high doses of methylcobalamin (500µg/kg) is found to improve compound muscle action potential in acrylamide induced neuropathy. Also some reports suggest the probable role of methylcobalamin in nerve regeneration and its clinical use for the same in peripheral neuropathy **[7]**. It was found that continous treatment with methylcobalamin showed to amileiorate effects on peripheral lesions in experimental diabetic neuropathy **[8]**. The objective of the study was to evaluate anti-hyperalgesic and neuroprotective effect of ultra high dose of methylcobalamin (500µg/kg) (MCA) after sciatic nerve lesion produced by partial sciatic nerve ligation **[9]** and sciatic nerve crush injury **[10]** in rats.

## Material and Methods

All procedures were conducted according to NIH animal care and use committee guidelines and all the experimental procedure used in this study were reviewed and approved by Institutional Animal Care and Use Committee of Poona College of Pharmacy, Pune, India **[10]**

**Animals:** Forty eight adult female Wistar rats (175-225g) were obtained from National Toxicology Centre, Pune, India. On arrival, the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2°C and relative humidity of 30-70%. A 12:12 light : dark cycle was followed. All animals had free access to water and standard pellet laboratory animal diet.

**Preparation of the drug solution MCA :-** Solution was prepared by dissolving methylcobalamin (Himedia) in distilled water. The drug solution was stored in air tight amber coloured bottles at room temperature in a cool and dry place away from moisture and sunlight. The solutions were freshly prepared at the time of dosing for daily administration. The volume of drug solutions were calculated based upon the body weight of the animal.

**Mononeuropathy caused by Partial Sciatic Nerve Ligation (PSNL) in Rats.**

The method of Seltzer (1990) was followed. The animals were divided into Control (vehicle treated), Sham(vehicle treated), Untreated, MCA treated.

**Preparation of animals:-**Under ether anesthesia and aseptic conditions the right sciatic nerve was exposed at high thigh level. In sham operated animals the nerve was left intact and the wound was closed with 2 muscle sutures and 3-4 skin sutures.

In experimental animals the sciatic nerve underwent partial injury. The dorsum of the nerve was carefully freed from surrounding connective tissues at a site near the trochanter just distil to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. Using an iris forceps the nerve was fixed in its place by pinching

the epineurium on its dorsal aspect, taking care not to press the nerve against underlying structures. A 4-0 silk suture was inserted into the nerve with 3/8 curved, reverse- cutting mini needle, and tightly ligated so that the dorsal 1/3 – ½ of the nerve thickness was trapped in the ligature. The wound was then closed as in sham operated rats. In all rats the left leg and sciatic nerve were untouched. The animals were allowed to recover after surgery. From day 2 onwards MCA (500µg/kg) treatment once daily, i.p. was started and continued for 30 days. The observations were recorded daily in the morning (between 10 am to 12 pm) and doses were administrated immediately afterwards

The effect of MCA was studied on the following parameters.

**Thermal hyperalgesia (TH):-** This was assessed using Ugo Basile Hot Plate Analgesiometer (at 55±1° C) **[12]**. Antinociceptive effects were determined according to the latency (in seconds) of limb withdrawal to the noxious thermal stimulation. Cut off value of planter test was set to 22 s to prevent limb injury. The pain thresholds were tested on day 0,2,4,6,8,10,12,14, 21 and 28.

**Motor function test (MFT)** :- Motor function was monitored by observation of spontaneous gait and hind paw posture **[13]**. Each animal was placed in a plastic box with plexiglass walls and allowed to habituate for at least 5 minutes before the observation period. One animal at a time was observed for 15 min (3 x 300s). Different positions of the lesioned hind paw were rated according to a numerical scale described by Attal et al (1991). The readings were observed on day 0, 2, 15 and 30.

**Mononeuropathy caused by Sciatic Nerve Crush Injury (SNCI) in Rats.**

The method of Bishofs et el (2004) was followed. The animals were divided into Control (vehicle treated), Sham(vehicle treated), Untreated, MCA treated.

**Preparation of animals:-** Under ether anesthesia and aseptic conditions the right sciatic nerve was exposed at high thigh level. In sham operated animals the nerve was left intact and the wound was closed with 2 muscle suture and 3-4 skin sutures.

In experimental animals the sciatic nerve underwent crush injury. The dorsum of the nerve was carefully freed from surrounding connective tissues at a site near the trochanter just distil to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. Using an iris forcep the nerve was fixed in its place by pinching

the epineurium on its dorsal aspect, taking care not to press the nerve against underlying structures. A blunt forcep was used to crush the nerve twice for a period of 30 s with an interval of 60 s in between. The wound was then closed as in sham operated rats. In all rats the left leg and sciatic nerve were untouched.

The effect of MCA was studied on the following parameters.

**Thermal hyperalgesia (TH):-** TH was assessed as described earlier on day 0,2.7.14.21 and 28.

**Motor function test (MFT)** :- MFT was assessed as described earlier on day 0,2,15 and 30.

**Motor Nerve Conduction Velocity (MNCV)** :- The experiment was performed on the same group of rats on day $15^{th}$ and $30^{th}$ day. Rats were anesthetized using thiopental sodium (50 mg/kg, i.p) for electrophysiological recording. MNCV was recorded by stimulating the sciatic and tibial nerves at sciatic and tibial notch respectively by a 0.1 ms square wave pulse delivered through a pair of monopolar needle electrodes (1.0 – 1.5 mA, 2.0 mV/D) through a stimulator. Responses were recorded from the indigital plantar muscles using Students Biopac data acquisition system (Santa Barbara, CA, USA).

## Statistical Analysis

Data was expressed as mean ± SEM of 6 animals in each group. To determine the statistical significance, ANOVA followed by Tukey-Kramer test (Instat/ Graphpad) was used. Differences between means were considered statistically significant if $p < 0.001$. For motor function test Kruskal-Wallis test followed by Dunn was used.

## Results

**Effect of Methylcobalamin (500µg/kg) on Thermal hyperalgesia in Partial sciatic nerve ligation (PSNL) model in rats:**

The latency (sec) of thermal hyperalgesia did not change significantly in normal animals. On the other hand, animals of untreated group showed a significant decrease in latency from day 2 (3.24 ± 0.13 s) to day 28 (3.52 ± 0.21 s)indicating feeling of pain when exposed to thermal stimuli. In the sham treated group, there was decrease in latency on day 2 (7.67 ± 0.32 s) only. Later on the animals did not exhibit decrease in latency. MCA treatment showed improvement in the latency from day 8 onwards (9.61 ± 0.19 s). Maximum reduction in latency was observed during day 4 (7.78 ± 0.11 s)and day 6 (8.55 ± 0.15 s) appeared to be due to gradual recovery after administration of MCA from day 2 (4.01 ± 0.13 s), non-significant change in latency observed from day 8 – day 28 (10.5 ± 0.16 s)indicate beneficial effect of MCA due to complete recovery from thermal algesia.

**Table 1. Effect of Methylcobalamin (500µg/kg) (MCA) on Thermal hyperalgesia in Partial sciatic nerve ligation (PSNL) model in rats**

| | ***Latency in seconds*** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Day 0*** | ***Day 2*** | ***Day 4*** | ***Day 6*** | ***Day 8*** | ***Day 10*** | ***Day 12*** | ***Day 14*** | ***Day 21*** | ***Day 28*** |
| Normal | 10.01 ±0.31 | 10.36 ±0.42 | 10.5 ±0.24 | 10.0 ±0.32 | 9.61 ±0.16 | 10.72 ±0.08 | 10.68 ±0.16 | 10.65 ±0.16 | 10.98 ±0.09 | 10.21 ±0.12 |
| Sham | 9.23 ±0.24 | 7.67± 0.32 | 10.0 ±0.26 | 9.65 ±0.17 | 9.03 ±0.18 | 10.25 ±0.14 | 10.48 ±0.14 | 10.54 ±0.25 | 10.61 ±0.08 | 10.43 ±0.11 |
| Untreated | 10.4 ±0.26 | 3.24± 0.13 | 3.2± 0.09 | 3.55± 0.12 | 3.64± 0.12 | 3.28± 0.09 | 3.82± 0.13 | 3.81± 0.16 | 3.21± 0.09 | 3.52± 0.21 |
| MCA | 11.52 ±0.17 | 4.01± 0.13 | 7.78± 0.11$^{\#}$ | 8.55***** ±0.15 | 9.61***** ±0.19 | 9.93***** ±0.18 | 10.58***** ±0.12 | 10.66***** ±0.24 | 10.73***** ±0.23 | 10.5***** ±0.16 |

***Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test.. p <0.001 = *. Data of MCA group was compared with that of untreated group. n=6***

**Effect of Methylcobalamin (500µg/kg) on Motor function test in Partial sciatic nerve ligation (PSNL) model in rats:**

MFT scores were 0 in all four groups on day 0. In the normal group these scores were not changed on subsequent days of observation. In the sham treated groups, the scores observed were less compared to that of MCA / untreated group. The untreated group showed increase in the progressive scores from day 2 (2.92 ± 0.08) to day 30 (3.43 ± 0.8) indicating the presence of pain in the animals. On the other hand in the MCA treated group, significant reduction in MFT score was observed on day 15 (1.46 ± 0.1) and day 30 (1.24 ± 0.09), indicating a gradual amelioration of neuropathic pain. **[Table 2]**

**Table 2. Effect of Methylcobalamin (500µg/kg) (MCA) on Motor function test in Partial sciatic nerve ligation (PSNL) model in rats**

| | ***Motor function test scores*** | | | |
|---|---|---|---|---|
| | ***Day 0*** | ***Day 2*** | ***Day 15*** | ***Day 30*** |
| Normal | 0.0 | 0.0 | 0.0 | 0.0 |
| Sham | 0.0 | 0.271 ± 0.05 | 0.087 ± 0.027 | 0.099 ± 0.02 |
| Untreated | 0.0 | 2.92 ± 0.08 | 2.98 ± 0.09 | 3.43 ± 0.8 |
| MCA | 0.0 | 2.86 ± 0.066 | 1.46***** ± 0.1 | 1.24***** ± 0.09 |

***Data expressed in Mean ± S.E.M. Kruskal Wallis followed by Dunn. p <0.001 = *. Data of MCA group was compared with that of untreated group. n=6***

**Effect of Methylcobalamin (500µg/kg) on Thermal hyperalgesia in Sciatic nerve crush injury (SNCI) model in rats:**

In normal animals, the pain latency did not show any remarkable change during the observation period of day 0 to day 28. In sham treated group reduction in pain latency or thermal hyperalgesia was observed on day 2 (9.95 ± 0.22 s) but not on subsequent days. The untreated group showed significant reduction of latency from day 2 (4.97 ± 0.19 s) to day 28 ( 4.78 ± 0.19 s) indicating presence of pain. In MCA treated group, on day 2 (5.18 ± 0.18 s) significant reduction of latency was observed which gradually increased on day 7 (9.41 ± 0.06 s) till day 28 (11.4 ± 0.05 s), indicating recovery from algesia induced by heat.

**Table 3. Effect of Methylcobalamin (500µg/kg) (MCA) on Thermal hyperalgesia in Sciatic nerve crush injury (SNCI) model in rats**

| | ***Latency in seconds*** | | | | | |
|---|---|---|---|---|---|---|
| . | ***Day 0*** | ***Day 2*** | ***Day 7*** | ***Day 14*** | ***Day 21*** | ***Day 28*** |
| Normal | 11.87 | 11.6 | 11.3 | 10.95 | 10.98 | 11.48 |
| | ±0.48 | ±0.49 | ±0.307 | ±0.33 | ±0.31 | ±0.20 |
| Sham | 11.91 | 9.95 | 10.75 | 11.4 | 11.3 | 11.38 |
| | ±0.32 | ±0.22 | ±0.23 | ±0.37 | ±0.20 | ±0.34 |
| Untreated | 11.92 | 4.97 | 5.26 | 5.23 | 4.95 | 4.78 |
| | ±0.20 | ±0.19 | ±0.11 | ±0.06 | ±0.09 | ±0.19 |
| MCA | 12.18 | 5.18 | 9.41* | 11.46* | 11.01* | 11.4* |
| | ±0.34 | ±0.18 | ±0.06 | ±0.2 | ±0.09 | ±0.05 |

***Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test. p <0.001 = *. Data of MCA group was compared with that of untreated group. n=6.***

**Effect of Methylcobalamin (500µg/kg) on Motor function test in Sciatic nerve crush injury (SNCI) model in rats:**

MFT scores were 0 in all four groups on day 0. In the normal group these scores were not changed on subsequent days of observation. In the sham treated groups, the scores observed were less compared to that of MCA / untreated group. The untreated group showed increase in the progressive scores from day 2 (2.92 ± 0.08) to day 30 (3.03 ± 0.07) indicating the presence of pain in the animals. On the other hand in the MCA treated group, significant reduction in MFT score was observed on day 15 (1.05 ± 0.02) and day 30 (1.048 ± 0.02), indicating a gradual amelioration of neuropathic pain.

**Table 4. Effect of Methylcobalamin (500µg/kg) (MCA) on Motor function test in Sciatic nerve crush injury (SNCI) model in rats**

| | ***Motor function test scores*** | | | |
|---|---|---|---|---|
| | ***Day 0*** | ***Day 2*** | ***Day 15*** | ***Day 30*** |
| Normal | 0.0 | 0.0 | 0.0 | 0.0 |
| Sham | 0.0 | 0.271 ± 0.05 | 0.087 ± 0.027 | 0.099 ± 0.02 |
| Untreated | 0.0 | 2.92 ± 0.08 | 3.16 ± 0.09 | 3.03 ± 0.074 |
| MCA | 0.0 | 2.99 ± 0.08 | 1.05***** ± 0.023 | 1.048***** ± 0.022 |

***Data expressed in Mean ± S.E.M. Kruskal Wallis followed by dunn. p <0.001 = *. Data of MCA group was compared with that of untreated group. n=6.***

**Effect of Methylcobalamin (500µg/kg) on Motor nerve conduction velocity in Sciatic nerve crush injury (SNCI) model in rats:**

The MNCV in the normal animals as well as sham treated animals did not significantly change on day 30 (53.01 ± 3.99 m/s, 47.86 ± 2.59 m/s respectively) )as compared to day 15 (52.75 ± 2.68 m/s, 47.19 ± 3.85 m/s respectively). The untreated group showed reduction in MNCV on day 30 (13.87 ± 0.58 m/s) as compared to day 15 (15.29 ± 0.71m/s) indicating loss of nerve function due to crush injury. In MCA treated group the conduction velocity recorded on day 30 (47.63 ± 2.12m/s) was more than that on day 15 (38.53 ± 2.69 m/s) indicating improved conduction due to drug treatment. **[Table 5]**

**Table 5**. **Effect of Methylcobalamin (500µg/kg) (MCA) on Motor nerve conduction velocity in Sciatic nerve crush injury (SNCI) model in rats**

| | ***Conduction Velocity in m/s*** | |
|---|---|---|
| ***Group*** | ***15$^{th}$ day MNCV (m/s)*** | ***30$^{th}$ day MNCV (m/s)*** |
| Normal | 52.75 ± 2.68 | 53.01 ± 3.99 |
| Sham | 47.19 ± 3.85 | 47.86 ± 2.59 |
| Untreated | 15.29 ± 0.71 | 13.87 ± 0.58 |
| MCA | 38.53$^{*}$ ± 2.69 | 47.63$^{*}$ ± 2.12 |

***Data expressed in Mean ± S.E.M. ANOVA followed by Tukey test. p <0.001 = *. Data of MCA group was compared with that of untreated group. n=6.***

## Discussion

Patients with peripheral nerve injuries occasionally experience chronic pain. This phenomenon is classified as neuropathic pain. Recently several animal models of neuropathic pain have been evaluated and there appear to be some similarities between these models and the clinical features of human patients. **[9, 14]**. The majority of currently used neuropathic pain models share alterations in hind limb cutaneous sensory thresholds following partial injury to a peripheral (usually sciatic) nerve as a common feature. In particular, demonstration of hyperalgesia to noxious thermal stimuli and allodynia to cold and mechanical stimuli are used as outcome measures. Three most commonly used models are the chronic constriction injury (CCI) of sciatic nerves, the partial sciatic nerve ligation model (PSNL), and the spinal nerve ligation model (SNL). **[4]** Nerve crush might be considered an extreme version of compressive nerve injury with an enhanced degree of vasa nervorum disruption **[15]**. Three factors are thought to cause hyperalgesia in the sciatic nerve ligation model, first is the ectopic discharge generated from injured axons, second is release of cytokines from the inflammatory cells around the injured nerve and third is plastic changes in the sensory pathways to the spinal cord and brain **[14]**.

There is evidence that severe peripheral nerve ischemia from vascular ligation damages endothelium, resulting in swelling, luminal narrowing and no re-flow. Although compression may result in temporary circulatory arrest it is unclear wheather this insult permanently injures nerve micro vessels. Comprehensive ischemia if maintained long enough, might induce no re-flow, as in the ligation experiments **[15]**.

In a serious trauma like crush, a short period of localized total or subtotal ischemia is followed by evident increase in endoneural fluid pressure and impairment of the normal capillary blood flow in the endoneurium. These events results in the release of the endogenous chemical mediators, increase in vascular permeability and impairment of blood nerve barrier. Endothelial and intraneural edema with inflammatory response follows this process **[5]**. The peripheral nerve responds to trauma by an inflammatory reaction with increased vascular permeability and intraneural edema, local ischemia in the tissue causes metabolic impairment, which in turn allows the production of the toxic

oxygen metabolites such as superoxide anion, hydrogen peroxide and hydroxyl radicals by the polymorphoneuclear leucocytes that infiltrate the lesion site. Free radicals and cytokines which are responsible for cell damage are released from neutrophills **[6]**.

Results of the present study indicated that in PSNL and SNCI models of mononeuropathy, MCA (500µg/kg, I.P, 30 days) gradually reduced the pain latency(sec) produced by thermal stimulus **[Table 1,3]**. The onset was observed after 4 days of treatment in PSNL and after 7 days in SNCI model. Further continuation of treatment resulted in restoration of latency to heat stimuli to pre surgery period. The present study was further extrapolated to MFT, a behavioral parameter of assessing mononeuropathy in PSNL and SNCI. MCA (500µg/kg, I.P, 30 days) treatment improved stance and pain endurance caused by nerve injury **[Table 2,4]**. After 15 days of treatment the pain scale was not significantly different compared to that of day 0, indicating amelioration in pain. Reduction in MNCV is an indication of neuropathic pain, while restoration of passage of impulse indicate an increase in conduction velocity. MNCV in untreated group was reduced while in MCA (500µg/kg, I.P, 30 days) treated group increase in the conduction velocity was observed which after 30 days of treatement was equal to that of sham **[Table 5]**.

Intravenous methylcobalamin treatment is a safe and potentially beneficial therapy for neuropathy is chronic hemodialysis patients **[16]**. Methylcobalamin has established therapeutic uses in the treatment of diabetic neuropathy. Also methylcobalamin treated rats showed significantly faster compound muscle action potential recovery in acrylamide induced neuropathy **[7].** Continous treatment with methylcobalamin had an ameliorative effect on the peripheral nerve lesions in STZ induced diabetic neuropathy in rats **[8].**

Our investigation suggest that methylcobalamin (500µg/kg, I.P, 30 days) is effective against TH in PSNL and SNCI models of mononeuropathy in rats. The results conclude that methylcobalamin can be used in improving nerve conduction velocity which opens a possibility of exploring the potential of ultra high dose of methylcobalamin ((500µg/kg) in the treatment of heat or crush injuries

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