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Effects of *Spirulina platensis* on neuropathic pain in Wistar rats

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Abstract

Background: In treatment of neuropathic pain, conventional analgesics showed various adverse effects. *Spirulina platensis* (*Sp*), a medicinal herb, shown to possess several beneficiary biological activities including analgesic, anti-inflammatory and anti-ulcerogenic potential. **Objectives:** To assess the effect of *Sp* on neuropathic pain in Wistar rats and also to assess the involvement of ATP sensitive potassium channel (K_{ATP}) as its possible underlying mechanism of action. **Methods:** For this experimental study 120 Wistar rats of both sexes (200 ± 50 gm body weight) were grouped into control [normal saline (NS) 5 ml/kg/day], sham control [sham surgery + NS], CCI control [Chronic constriction injury to sciatic nerve (CCI) + NS], *Sp* experimental [CCI + *Sp* 400 mg/kg/day], Gli experimental [CCI + *Sp* (400 ml/kg/day) + glibenclamide (15 mg/kg)] groups. *Sp* and NS were administered orally once daily for consecutive 21 days and single dose of glibenclamide was given intraperitoneally. Then again on the basis of neuropathic pain evaluation tests, all the groups were subdivided into 'a' (for walking track analysis), 'b' (for cold tail immersion test), 'c' (for von Frey test), 'd' (for hot plate test). The statistical analysis was done by one-way ANOVA followed by Bonferroni post hoc test, where $P \leq 0.05$ was considered as statistically significant. **Results:** In this study, *Sp* showed significantly

($P \leq 0.001$) higher sciatic functional index, tail flick latency, paw withdrawal threshold and reaction time in *Sp* experimental rats compared to those of CCI control rats. In addition, there were significant ($P \leq 0.001$) differences in the above-mentioned variables between rats of *Sp* experimental group and Gli experimental group. **Conclusion:** From the present study it could be concluded that, *Spirulina platensis* prevents the development of neuropathic pain in Wistar rats through opening of K_{ATP} channel.

Keywords: Neuropathic pain, *Spirulina platensis*, glibenclamide, walking track analysis, cold tail immersion test, von Frey test, hot plate test.

Introduction

Neuropathic pain, a chronic morbidity, arises from injured axons as well as from intact nociceptors which results in abnormal sensory signs, allodynia (pain response to low threshold stimulus) and hyperalgesia (increased response to noxious stimuli). According to World Health Organization (WHO) about 22% of the world's primary care patients suffer from chronic neuropathic pain¹. However, different mechanisms have been proposed as the causes of this neuropathic pain, such as, release of inflammatory cytokines, increased receptor signaling, intracellular Ca^{++} ion accumulation within the injured neuron, glial cell activation as well as ectopic discharge in lesioned fibers^{2,3}, voltage gated calcium channel activation⁴, increased expression of voltage gated sodium channel⁵ and opening of ATP sensitive potassium channel (K_{ATP})⁶ in the membranes of nerve fibers.

For alleviation of this agonizing neuropathic pain, tricyclic antidepressants (i.e. amitriptyline, nortriptyline, imipramine) as well as anticonvulsants (i.e. phenytoin, carbamazepine, gabapentin, lamotrigine and topiramate) are the present treatment regimens⁷. To minimize the adverse effects associated with regular use of these classes of drugs, many researches have been carried out on treatment schedule of neuropathic pain with different herbal products⁸⁻¹². Within

them *Spirulina* (world's largest natural protein source) has been suggested as an important medicinal herb^{13,14}.

Along with its high nutritional value, *Spirulina platensis* (*Sp*) has also showed immunomodulatory¹⁵, anti-inflammatory^{11,15}, cardioprotective¹⁶, renoprotective¹⁷, antihyperlipidemic¹⁸, antioxidant¹⁹, anticancer²⁰, protective against heavy metals²¹, anti-diabetic²², hepatoprotective²³, neuroprotective²⁴ as well as analgesic¹¹ effects in different animal models. Besides these preclinical studies, *Sp* was also reported to be preventive of allergy and rhinitis²⁵, preventive of anaemia²⁶ and cholesterol-lowering²⁷ agents in different human studies. Moreover, in perspective of pain, this medicinal alga was shown to affect nociceptive and inflammatory pain^{11,14} as well as neuropathic pain^{11,12,19} in different animal models.

In addition, *Sp* was shown to prevent neuropathic pain by its antioxidative capacity¹⁹. But as far as we searched, no study was found to explore the role of K_{ATP} channel in analgesic mechanism of this medicinal herb. Based on this background, the present study has been designed to evaluate the effect of *Sp* on neuropathic pain in Wistar rat and also to evaluate the role of K_{ATP} channel in analgesic action of this herb.

Methods

The study was conducted in the Pain laboratory of Department of Physiology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, and Department of Pharmacology, Faculty of Veterinary Science, Bangladesh Agriculture University, Mymensingh, from March 2019 to February 2020, with prior approval from the Institutional Review Board of BSMMU.

Procurement and maintenance of animals

Total 120 rats (200 ± 50 gm) of both sexes²⁸ were collected from the central animal house of BSMMU and were housed in specially built plastic cages with 6 rats per cage under a 12/12 hour light/dark cycle²⁹. As the thermoneutral zone for rodents is around 27 to 28°C³⁰, the ambient room temperature was maintained at 27°C. Free access to standard laboratory food³¹ and cooled boiled water *ad libitum* were provided to all rats during acclimatization. All the experiments were performed at daytime between 08:00 and 16:00 hours to avoid circadian influences in accordance with the international guidelines on the use of laboratory animals of International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

Dose schedule

Four hundred (400) mg/kg body weight³² powder of *Sp* [Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh] was dissolved in normal saline (NS) and 15 mg/kg body weight³³ powder of glibenclamide (United Pharma Industries Co Ltd. China) was dissolved in Carboxy Methylcellulose (Beximco Pharma Ltd. Bangladesh).

Experimental design

On the basis of treatments, all rats (n=120) were grouped into control [normal saline (NS) 5 ml/kg], sham control [sham surgery + NS], CCI control [Chronic constriction injury to sciatic nerve (CCI) + NS], *Sp* experimental (CCI + *Sp* 400 mg/kg), Gli experimental [CCI + *Sp* (400 ml/kg) + glibenclamide (15 mg/kg)] groups. *Sp* and NS were administered orally once daily for consecutive 21 days and single dose of glibenclamide was given intraperitoneally just 10 minutes before every test. Then again on the basis of neuropathic pain evaluation tests, all the groups were subdivided into ‘a’ (for walking track analysis), ‘b’ (for cold tail immersion test), ‘c’ (for von Frey test), ‘d’ (for hot plate test) as shown in figure 1.

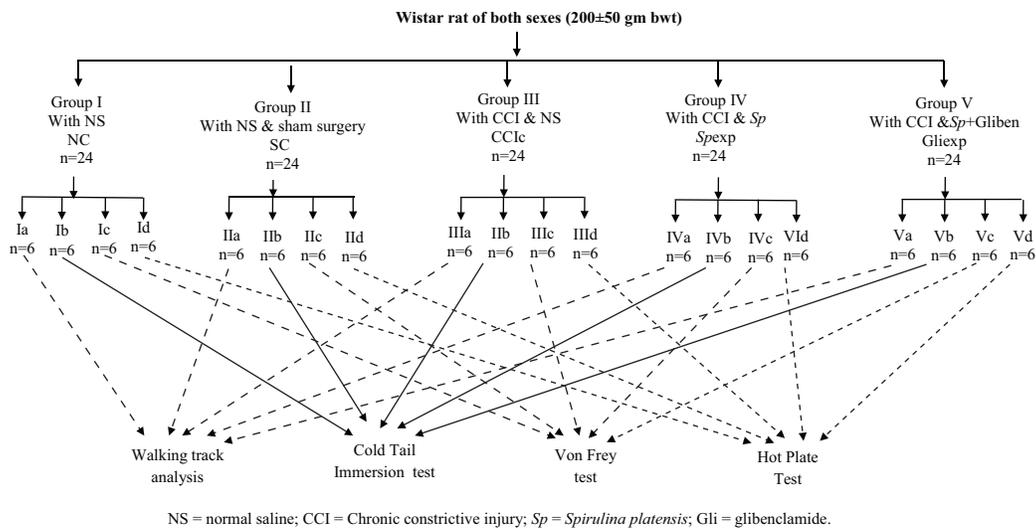


Figure 1: Experimental design and animal grouping.

Chronic Constrictive Injury of sciatic nerve (CCI)

To produce neuropathic pain, chronic constrictive injury of sciatic nerve (CCI) was performed according to Bennett and Xie (1988)³⁴. At first, rat was anesthetized, using intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture. After shaving and sterilization, proper positioning was done. Incision was given on the left thigh 3 to 4 mm below the femoral head and the sciatic nerve was exposed. Four (4) loose ligations were made around the common sciatic nerve at 1 mm intervals. Constriction of the nerve was minimal, to prevent arresting of the epineural blood flow. Finally, the muscle layer and the skin were closed. In the sham group, after exposing the sciatic nerve only, muscle and skin were sutured.

Walking track analysis

Walking track analysis was performed according to previously published procedures^{32,35}. Six (6) rats from each group (total 30) were separated for walking track analysis. All of the rats were acclimatized individually for consecutive 7 days to walk freely on a smooth, flat surface covered by a sheet of ink absorbing paper guarded by wooden plate. This test was done every 4 days interval after CCI induction. On the day of experiment just one hour after the treatment each rat was kept individually in a above mentioned track on a sheet of ink absorbing paper, with their hind paws dipped in blue ink. Every time 3 to 4 foot prints of each foot were marked and a measuring scale was used to take the measurements as mentioned below. Then sciatic functional index (SFI) was calculated as follows: $SFI = [-38.3 \times (EPL-NPL)/NPL] + [109.5 \times (ETS-NTS)/NTS] + [13.3 \times (EIT-NIT)/NIT] - 8.8$; where NPL = normal print length, EPL = experimental print length, NTS = normal toe spread, ETS = experimental toe spread, NIT = normal intermediate toe spread, and EIT = experimental intermediate toe spread.

Cold tail immersion test

Cold tail immersion test procedure was performed according to the methods of previous publications^{36,37}. Six (6) rats from each group

were separated for this test and instrumental acclimatization was done for consecutive 7 days. Then during experiment, each rat was kept individually in the plexiglass mechanical restrainer for 5 minutes to adjust to the cage environment, with the tail hanging freely. Then distal 5 cm of the freely hanging tail of the rat was immersed into cold water maintained at 10°C and latency period of the tail withdrawal (tail-flick) was recorded. The mean of the measurements was obtained from three similar successive maneuvers, performed at 5 minutes intervals and was recorded as the baseline latency. One hour after the last dose of the treatment (NS/ *S. platensis*), another tail immersion test was done. Here the mean of 3 tail withdrawal latencies at 5 minutes interval was noted as test latency (TL). To minimize tissue damage, a maximum latency of 20 seconds was considered as cut off time. The antinociceptive effect was expressed as percentage of maximum possible effect (% MPE) as follows: $[(TL-BL) / (Cut\ off\ time-BL)] \times 100$.

Von Frey test

Von Frey test procedure was performed according to the previously described method^{38,39}. Six (6) rats from each group (Total 30) were recruited for Von Frey test and instrumental acclimatization was done for 7 consecutive days. This test was done on every 4 days interval after CCI induction. At the day of experiment, just 1 hour after the treatment each rat was placed individually on the wide gauge wire mesh surface. Then the calibrated Von Frey filaments (VFF) was touched in an ascending order on planter surface of left hind paws of the rat between first and second metatarsal approximately 1cm proximal to the ankle joint. Each VFF (of varying tensile strengths of 2 to 18 gm) was applied three times at 30 seconds intervals and the number of hind paw withdrawal was recorded. The next larger VFF was applied unless paw withdrawal occurred in at least two of the three. If the rat fails to withdrawal its paw at maximum force of 18 gm, then no more VFF (of increased tensile strength) was applied, to prevent tissue injury.

Hot plate test

Hot plate test procedure was performed according to the previously described

method^{28,40}. Six (6) rats from each group (total 30) rats were separated for Hot plate test. Along with laboratory acclimatization these rats were also further acclimatized in the instrument for 1 hour daily for 7 consecutive days. On the experimental day, the hot plate was heated at a temperature of $54 \pm 0.5^\circ\text{C}$ and the rat was placed on heated surface. A stop clock was started as the rat placed on the hot plate till the first paw licking or trying to jump out. A cut off period of 20 sec was set to avoid any damage to the paw. This was done at interval of 30, 60, 90 min respectively after *S. platensis* administration. The analgesia was expressed as Percentage of maximal possible effect (%MPE) as follows: $[(\text{TL-BL}) / (\text{Cut off time-BL})] \times 100$.

Statistical analysis

Results were expressed as mean \pm SEM. Data were analyzed in SPSS (version 23.0) using one-way ANOVA followed by Bonferroni post hoc test, where $P \leq 0.05$ was considered as statistically significant.

Results

Effect of *Sp* on sensorimotor dysfunction in walking track analysis.

As shown in Figure 2A. *Sp* increased the sciatic functional index (SFI) significantly ($P \leq 0.001$) in *Spexp* rats (CCI plus *Sp*) in comparison to that of CCIc rats (only CCI) on day 9, 14, 19 and 24 of experiment. Moreover, there was significant ($P \leq 0.001$) decrement of SFI in Gli exp rats (CCI plus *Sp* plus Gli) in comparison to that of *Spexp* rats on day 14, 19 and day 24.

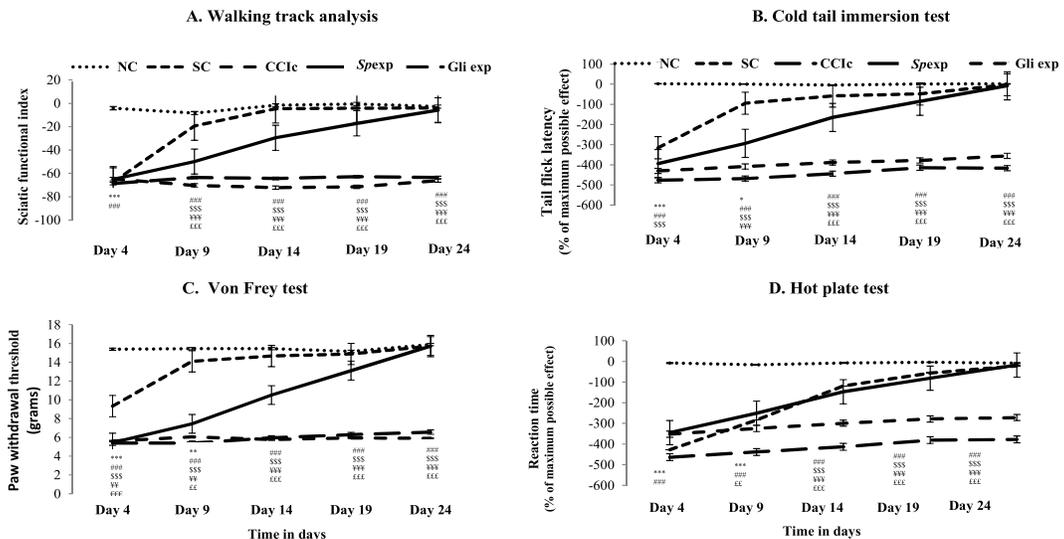


Figure 2: A. Sciatic functional index in rats with walking track analysis, B. Tail withdrawal latency in rats with cold tail immersion test, C. Paw withdrawal threshold in rats with Von Frey test, D. Reaction time in rats with hot plate test. Each line symbolizes mean \pm SEM for 6 rats. NC = rats with oral normal saline (NS, 5 ml/kg) for consecutive 21 days; SC = rats with sham surgery followed by oral NS (5 ml/kg); CCIc = rats with chronic constrictive injury to sciatic nerve (CCI) followed by oral NS (5 ml/kg) for consecutive 21 days; *Spexp* = rats with CCI followed by oral *Sp* (400 mg/kg) for consecutive 21 days; Gliexp = rats with CCI followed by oral *Sp* (400 mg/kg) for consecutive 21 days and also with single dose of intraperitoneal glibenclamide (15 mg/kg) 10 minutes before *Sp* on the day of experiment. * = NC vs SC, # = NC vs CCIc, \$ = SC vs CCIc, ¥ = CCIc vs *Spexp*, • = CCIc vs Gliexp, and £ = *Spexp* vs Gliexp. Single symbol = significant at $p \leq 0.05$, double symbol = significant at $p \leq 0.01$, triple symbol = significant at $p \leq 0.001$.

Effect of Sp on cold allodynia in cold tail immersion test

As shown in Figure 2B, Sp increased the tail flick latency significantly ($P \leq 0.001$) in Spexp rats (CCI plus Sp) in comparison to that of CCIc rats (only CCI). Moreover, there was significant ($P \leq 0.001$) decrement of tail flick latency in Gli exp (CCI plus Sp plus Gli) rats in comparison to that of Spexp rats.

Effect of Sp on mechanical allodynia in Von Frey test.

As shown in Figure 2C, Sp increased the paw withdrawal threshold significantly in Spexp rats (CCI plus Sp) in comparison to that of CCIc rats (only CCI) on day 9 ($P \leq 0.01$), 14 ($p \leq 0.01$) and 19 ($P \leq 0.001$) and day 24 ($P \leq 0.001$) of experiment. Moreover, there was significant ($P \leq 0.001$) decrement of paw withdrawal threshold in Gli exp (CCI plus Sp plus Gli) rats in comparison to that of Spexp rats.

Effect of Sp on heat hyperalgesia in hot plate test.

As shown in Figure 2D, Sp increased the reaction time significantly in Spexp rats (CCI plus Sp) in comparison to that of CCIc rats (only CCI) on day 14 ($P \leq 0.05$), day 19 ($P \leq 0.001$) and day 24 ($P \leq 0.001$) of experiment. Moreover, there was significant ($P \leq 0.001$) decrement of reaction time in Gli exp (CCI plus Sp plus Gli) rats in comparison to that of Spexp rats on day 9 ($P \leq 0.05$), day 14 ($P \leq 0.001$), day 19 ($P \leq 0.001$) and day 24 ($P \leq 0.001$) of experiment.

Discussion

Pain management is quite a difficult task in clinical practice, where chronic neuropathic pain tends to exhibit a relatively poor response to traditional analgesics⁴¹. Moreover, wide range of adverse effects and several interactions with other drugs limit the use of traditional analgesics. These reasons triggered us to find a safer remedy for this unbearable morbidity. To measure the severity of neuropathic pain, various methods

have been used in animal models. Among them the walking track analysis is a quantitative method to analyze the functional impairment of sciatic nerve^{35,42}. It provides a noninvasive method of assessing the functional status of the sciatic nerve during the regeneration process. In addition, the cold tail immersion test is one of the most common thermal method⁴³ where tail withdrawal latency is measured for assessment of cold allodynia⁴⁴ which signifies mainly the changes above the spinal cord level in neuropathic rats. Von Frey test is a standard method for measurement of mechanical allodynia in neuropathic pain in rats⁴³. Here paw withdrawal threshold is the measuring variable^{45,46}. Hot plate test is another method for measurement of thermal allodynia in neuropathic pain in rats. It involves higher brain function and is considered to be a supraspinally organized response. The hot plate procedure constitutes a more global estimate of nociceptive reactivity because it represents a complex willed behavior rather than a simple reflex.

In our study, loose ligation around sciatic nerve might compress the nerve fiber causing nerve damage in the rats with CCI as evidenced by significant difference of SFI in walking track analysis, tail flick latency in cold tail immersion test, paw withdrawal threshold in von Frey test and reaction time in hot plate test, in comparison to those of normal rats and rats with sham surgery. Here the damaged cell released various inflammatory mediators including prostaglandin E₂ (PGE₂), interleukine-1 α (IL-1 α), tumor necrosis factor- α (TNF- α), Interleukin 6 (IL-6), bradykinin, substance P, nerve growth factor^{3,47}; augmenting chemotaxis of inflammatory cells (macrophage, microglia, monocyte, helper T cell). These mediators cause activation of various signaling pathways resulting in alterations in different ion channels (e.g. Na⁺, Ca²⁺, K⁺ channels), which causes release of glutamate, ATP and other mediators from activated central terminal of nociceptor causing prolonged post

synaptic depolarization of dorsal root ganglia (DRG)³. Besides this, accumulation of toxic reactive oxygen species (ROS) might cause membrane damage, glial activation and central sensitization⁴⁸ with subsequent intensification of pain transmission. However, all or any of the above mentioned mechanisms might cause increment of pain transmission along with sensorimotor dysfunction of sciatic nerve, cold allodynia, mechanical allodynia and heat hyperalgesia in the experimental rats with CCI induced neuropathic pain.

Furthermore, in the present study, *Sp* prevented the development of all these four aspects of neuropathic pain in rats with CCI plus *Sp*, as evidenced by significant difference of SFI in walking track analysis, tail flick latency in cold tail immersion test, paw withdrawal threshold in von Frey test and reaction time in hot plate test, in comparison to those of rats with only CCI (Figure 2). However, many mechanisms might be involved in this prevention of neuropathic pain by *Sp* as evidenced by prevention of sensorimotor dysfunction of sciatic nerve, cold allodynia, mechanical allodynia and heat hyperalgesia in the experimental rats with *Sp*. Such as, prevention of decrement of total antioxidative power in CCI induced neuropathic pain¹⁹, reduction of inflammatory mediators (IL-1 α , TNF- α , IL-6, PG)^{49,50} and downregulation of microglial activation directly³².

However, we have investigated another possible mechanism of action through which *Sp* exert its beneficial effects on neuropathic pain. As our study showed that, *Sp* could not prevent the development of all the four aspects of neuropathic pain when glibenclamide was administered in rats with CCI plus *Sp*. It has been well known that glibenclamide is a blocker of K_{ATP} channel⁵¹. Therefore, this finding indicated that the analgesic action of *Sp* could occur through opening of K_{ATP} channel. To the best of our knowledge, this study for the first time evaluated the involvement of K_{ATP} channel in prevention of neuropathic pain by *Sp*.

Conclusions

Spirulina platensis prevents the development of neuropathic pain in Wistar rats via opening of K_{ATP} channel. Although, glibenclamide, the agent used to block the K_{ATP} channel in the present study is a nonspecific one, therefore, further studies are recommended using specific K_{ATP} channel blocker to ascertain the present findings.

Conflict of Interest: none

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Department of Pharmacology, Bangladesh Agriculture University, Mymensingh.

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