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ORIGINAL ARTICLE

Attenuation of neuropathic pain by sodium butyrate in an experimental model of chronic constriction injury in rats



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KEYWORDS

chronic constriction injury;
inflammation;
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sciatic nerve ligation;
sodium butyrate

Background/Purpose: The present study was designed to investigate the potential of sodium butyrate, a histone deacetylase (HDAC) inhibitor, in chronic constriction injury (CCI)-induced neuropathic pain in rats.

Methods: Neuropathic pain was induced by placing four loose ligatures around the sciatic nerve. Acetone drop, Von frey hair, pin prick and hot plate tests were performed to assess cold allodynia, mechanical allodynia, and mechanical and heat hyperalgesia, respectively. The level of tumor necrosis factor (TNF)- α was measured in the sciatic nerve as an inflammatory marker.

Results: CCI was associated with the development of cold allodynia, mechanical allodynia, and mechanical and heat hyperalgesia, along with an increase in TNF- α level. Administration of sodium butyrate (200 and 400 mg/kg, oral) for 14 days in CCI-subjected rats significantly attenuated behavior related to injury-induced pain and the increase in TNF- α level.

Conclusion: It may be concluded that the anti-inflammatory actions mediated by sodium butyrate are responsible for its beneficial effects in neuropathic pain in rats.

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Introduction

Neuropathic pain has been described as the “most terrible of all tortures which a nerve wound may inflict” and arises as a

consequence of nerve injury either of the peripheral or central nervous system. Following peripheral nerve injury, a cascade of events in the primary afferents leads to peripheral sensitization, resulting in spontaneous nociceptor activity, decreased threshold, and increased response to suprathreshold stimuli.¹ Despite the recent advances in identification of peripheral and central sensitization mechanisms related to nervous system injury, the effective treatment of patients suffering from neuropathic pain remains a clinical challenge. Although numerous treatment

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options are available for relieving neuropathic pain, there is no consensus on the most appropriate treatment.² Consequently, there is still a considerable need to explore novel treatment modalities for neuropathic pain management.

HDACs (also called lysine deacetylases) are a class of enzymes that regulate the gene expression by removing the acetyl group from ϵ -N-acetyl lysine amino acid present on histone proteins.^{3,4} Several studies have suggested the upregulation of HDAC in different inflammatory diseases, including polycythemia vera, essential thrombocythemia, rheumatoid arthritis, neuroblastoma, and pancreatic cancer.^{5–7} Accordingly, HDAC inhibitors are proposed to have significant therapeutic potential as anti-inflammatory and immunosuppressive drugs.⁸ Preclinical studies have shown that HDAC inhibitors produce beneficial effects in various pathological conditions such as rheumatoid arthritis,⁹ Rubinstein–Taybi syndrome, Rett syndrome, Friedreich’s ataxia, Huntington’s disease and multiple sclerosis,¹⁰ and acute central nervous system injury including ischemic and hemorrhagic stroke.¹¹

It has been suggested that increased expression of HDAC within the superficial dorsal horn is key to maintenance of persistent pain.¹² The pharmacological inhibition of class IIa HDAC in the spinal cord has been shown to attenuate inflammatory complete-Freund’s-adjuvant-induced thermal hyperalgesia in rats.¹³ Sodium valproate has been shown to produce beneficial effects in different neuropathic pain conditions, including those due to chemotherapeutic agents, and its pain-attenuating effects have been at least partly attributed to HDAC inhibition.¹⁴ Sodium butyrate is a noncompetitive inhibitor of HDAC¹⁵ and it selectively inhibits subtypes I and IIa.¹⁶ The present study was designed to investigate the neuropathic-pain-attenuating potential of sodium butyrate in chronic constriction injury (CCI)-induced neuropathic pain in rats.

Materials and methods

Experimental animals

Sprague–Dawley rats of either sex weighing 200–250 g (procured from Punjab University, Chandigarh, India) were used in the present study. They were housed in the animal house with free access to water and a standard laboratory chow diet (Kisan Feeds, Mumbai, India). The rats were exposed to normal cycle of 12 hours light and 12 hours dark. The experimental protocol was duly approved by the Institutional Animals Ethics Committee (IAEC) and care of 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 Forests, Government of India (Reg. No. – 107/1999/CPCSEA).

Drugs and reagents

Sodium butyrate was obtained from Sigma–Aldrich (St Louis, MO, USA). Bovine serum albumin and Folin–Cicalteu phenol reagent were obtained from S.D. Fine (Mumbai, India). All the chemicals used in the present study were of analytical grade. The tumor necrosis factor (TNF)- α assay kit was procured from RayBiotech (Norcross, GA, USA).

Induction of neuropathy by CCI

Peripheral neuropathy was induced by CCI as described by Bennett and Xie with slight modification,¹⁷ using silk 4-0 sutures instead of chromic gut sutures, because it has been documented that the latter initiate inflammatory reactions in the sciatic nerve.¹⁸ The rats were deeply anesthetized with chloral hydrate (350 mg/kg, intraperitoneal). The hair on the lower back and thighs of the rats was shaved, and the skin was sterilized with 0.5% chlorhexidine. The skin of the lateral surface of the left thigh was incised and a cut made directly through the biceps femoris muscle to expose the sciatic nerve. Once exposed, the sciatic nerve was ligated with silk 4-0 thread at four sites with a 1-mm gap. The ligatures were loosely tied until a short flick of the ipsilateral hind limb was observed. The muscle and skin were closed in two layers with the use of thread, and topical antibiotics were applied. All surgical procedures were carried out under normal sterile conditions. As a result of the distinct development of postural defects in the paws of CCI control animals, the behavioral studies were not blinded for comparison between the normal controls, sham controls, and CCI control groups. However, for all other groups the behavioral tests were blinded.

Behavioral examination

Paw cold allodynia (acetone drop test)

Cold allodynia was assessed by spraying 100 μ L of acetone onto the surface of the rat paw (placed over a wire mesh), without touching the skin. The response of the rat to acetone was noted for 20 seconds and was graded on a 4-point scale as defined by Flatters and Bennett¹⁹ (0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking; and 3, repeated flicking of the paw with licking of the paw). Acetone was applied three times to the hind paw, with a gap of 5 minutes between the acetone applications, and the individual scores noted at 20-second intervals were added to obtain a single score over a cumulative period of 1 minute. The minimum score was 0 and the maximum possible score was 9.

Mechanical hyperalgesia (pin prick test)

Mechanical hyperalgesia was assessed by the pinprick test, as described by Erichsen and Blackburn-Munro.²⁰ The surface of the injured hind paw was touched with the point of a bent gauge needle (at 90° to the syringe) at intensity sufficient to produce a reflex withdrawal response. The paw withdrawal duration was recorded in seconds and the normal quick reflex withdrawal response was given the value of 0.5 seconds.

Paw heat hyperalgesia (hot plate test)

The thermal nociceptive threshold, as an index of thermal hyperalgesia, was assessed by the Eddy’s hot plate, maintained at a temperature of $52.5 \pm 1.0^\circ\text{C}$. The rat was placed on the hot plate and withdrawal latency, with respect to licking of the hind paw, was recorded in seconds. The cut-off time of 15 seconds was maintained.²¹

Determination of mechanical allodynia by Von Frey hair test

Mechanical allodynia (non-noxious mechanical stimuli) was assessed as described by Chaplan *et al.*²² Calibrated nylon filaments (Von Frey hairs), in terms of different bending forces, were applied to the midplantar surface of left hind paw. The filaments were applied 10 times, starting with the softest and continuing in ascending order of stiffness. A brisk withdrawal of the left hind limb was considered a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal threshold of the left hind paw five times out of 10 trials, that is, a 50% response.

Biochemical estimations

Cytokine and protein assay

All the animals were sacrificed and the sciatic nerve samples (7–8 mm long and taken along with the shaft of the femur before sciatic trifurcation), were isolated immediately for biochemical analysis. The sciatic nerve was homogenized in phosphate-buffered saline, pH 7.4, and thereafter, the homogenates were processed immediately for centrifugation at 1500g, at 4°C for 10 minutes to obtain the supernatant for protein and TNF- α estimation.

Estimation of protein content

The protein concentration in the sciatic nerve was estimated according to the method of Lowry *et al.*,²³ using bovine serum albumin as a standard.

Estimation of TNF- α

TNF- α was estimated with a commercially available enzyme-linked immunosorbent assay (ELISA, SUNOSTIK-SPR-960 ELISA READER, Sunostik Medical Technology Co., Ltd, Jilin, Changchun, China). The concentration of TNF- α was expressed in pg/mg of protein.

Experimental protocol

Eight groups of six rats were used in the present study. Group I: normal control. Rats were not subjected to any treatment and were kept for 14 days. The behavioral tests were performed on Day 0, Day 7, and Day 14. On Day 14, the animals were sacrificed and the biochemical estimations were carried out. Group II: sham control. Rats were subjected to a surgical procedure to expose the left sciatic nerve on Day 1, without any nerve ligation. The behavioral tests were done on Day 1 (before surgery), Day 7, and Day 14, and the biochemical analysis was done as described for Group I. Group III: CCI. Rats were subjected to a surgical procedure to expose and ligate the left sciatic nerve on Day 1 as described above. The behavioral tests and biochemical analysis were done as described for Group II. Groups IV, V, and VI: sodium butyrate in CCI. Sodium butyrate [100 mg/kg, 200 mg/kg and 400 mg/kg, orally (p.o.) by syringe feeder] was administered to rats subjected to CCI, starting from Day 1 (30 minutes prior to anesthesia for surgery) to Day 14. The behavioral tests and the biochemical analysis were done as described for Group II. Group VII: vehicle-treated rats with CCI. Saline (1 mL/kg, p.o. by syringe feeder) was administered to rats subjected to CCI, from Day 1 (30 minutes prior to anesthesia for surgery) to Day 14. The behavioral tests and the biochemical analysis were done as described for Group II. Group VIII: sodium butyrate *per se*. Sodium butyrate (400 mg/kg, p.o.) was administered to normal rats for 14 days. The behavioral tests and the biochemical analysis were done as described for Group II.

Statistical analysis

The results were expressed as the mean \pm standard error of the mean. The data from the behavioral tests were analyzed using two-way analysis of variance (ANOVA)

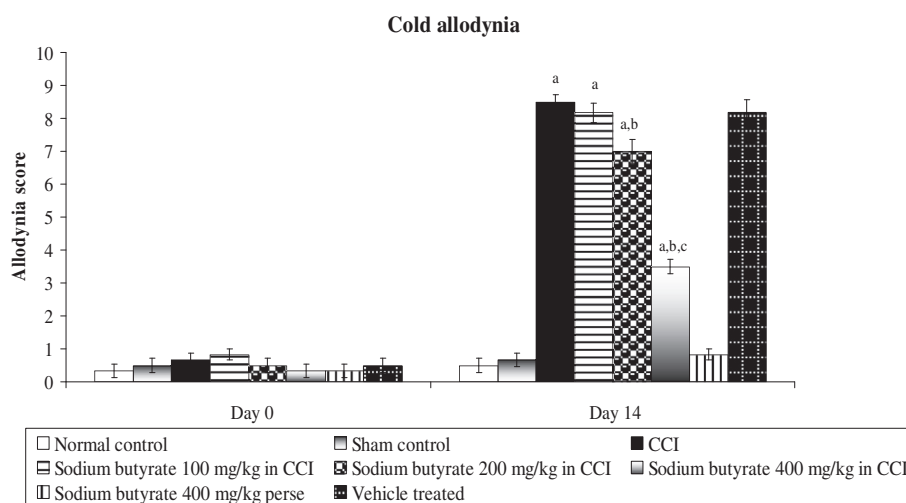


Figure 1 Effect of sodium butyrate on CCI-induced paw cold allodynia assessed by acetone drop test. Values are given as mean \pm standard error of the mean, $n = 6$ rats per group. Two-way analysis of variance followed by Bonferonni's *post hoc* test, $F(1, 80) = 1149.43$, for days, $p < 0.0001$ and $F(7, 80) = 119.34$ for treatment, $p < 0.0001$. ^a $p < 0.05$ versus sham control corresponding to same day. ^b $p < 0.05$ versus CCI corresponding to same day. ^c $p < 0.05$ versus sodium butyrate 100 mg/kg in CCI corresponding to same day. CCI = chronic constriction injury.

followed by Bonferonni's *post hoc* test, using GraphPad Prism version 5.0 (San Diego, California, USA). The data from the biochemical tests were analyzed using one-way ANOVA followed by Tukey's multiple range test. A p value < 0.05 was considered to be statistically significant.

Results

Effect of sodium butyrate on hyperalgesia and allodynia in CCI-induced neuropathic pain

CCI resulted in significant development of cold allodynia (Fig. 1), mechanical allodynia (Fig. 2), mechanical hyperalgesia (Fig. 3), and heat hyperalgesia (Fig. 4), as compared to the sham group, assessed by acetone drop test, Von Frey hair test, pin prick test, and hot plate test, respectively. Administration of sodium butyrate (200 mg/kg and 400 mg/kg, *p.o.*), for 14 days, significantly attenuated CCI-induced hyperalgesia and allodynia on Day 7 and Day 14. However, the pain symptoms were significantly higher on Day 14 as compared to Day 7 (data not shown). Administration of sodium butyrate (400 mg/kg, *p.o.*) did not modulate behavioral function in normal rats. Administration of vehicle did not alter CCI-induced hyperalgesia and allodynia.

Effect of sodium butyrate on TNF- α in CCI-induced neuropathic pain

CCI significantly elevated TNF- α level in the sciatic nerve on Day 14 as compared to that in sham control rats. Administration of sodium butyrate (200 mg/kg and 400 mg/kg, *p.o.*) significantly attenuated CCI-induced increase in TNF- α level. *Per se* administration of sodium butyrate (400 mg/kg *p.o.*) and the vehicle did not affect TNF- α in normal and CCI-subjected rats, respectively (Fig. 5).

Discussion

The CCI model is the most commonly used animal neuropathic pain model of nerve-damage-induced allodynia/hyperalgesia.^{24,25} In this model, neuropathic pain is induced by entrapping the sciatic nerve through four loose ligatures and the model shares the pathophysiology of carpal tunnel syndrome in humans due to entrapment of the median nerve in the narrowing tunnel. Furthermore, this model has also been suggested to share the pathophysiology of complex regional pain syndrome in humans.^{24,26} In the present study, CCI led to a significant development of cold allodynia, mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia assessed on Day 7 and Day 14 after surgery. In our previous studies, the peak behavioral alterations were reported on Day 14 day after the nerve injury in the CCI model.²⁷

In the present investigation, administration of sodium butyrate (200 mg/kg and 400 mg/kg, *p.o.*) for 14 days significantly attenuated CCI-induced behavioral alterations including paw cold allodynia, mechanical allodynia, and mechanical and heat hyperalgesia. Several pharmacological studies have shown the beneficial effects of sodium butyrate in different inflammatory conditions, such as a permanent ischemic model of stroke in rats, acute lung injury during cecal ligation, and puncture-induced polymicrobial sepsis.^{28,29} However, this is believed to be the first study showing the beneficial effects of sodium butyrate in CCI-induced neuropathic pain in rats. In the present study, drug was administered for 14 days after the nerve injury, therefore suggesting the therapeutic effect of sodium butyrate in the CCI model. Sodium butyrate is a noncompetitive inhibitor of HDAC¹⁵ and it selectively inhibits multiple subtypes of class I and IIa.¹⁶ Previous studies have suggested the key role of HDAC IIa in inflammatory pain because complete-Freund's-adjuvant-induced thermal hyperalgesia is inhibited by HDAC IIa inhibition.¹³ Accordingly, it may be tentatively proposed that sodium-butyrate-mediated HDAC IIa inhibition is probably responsible for its

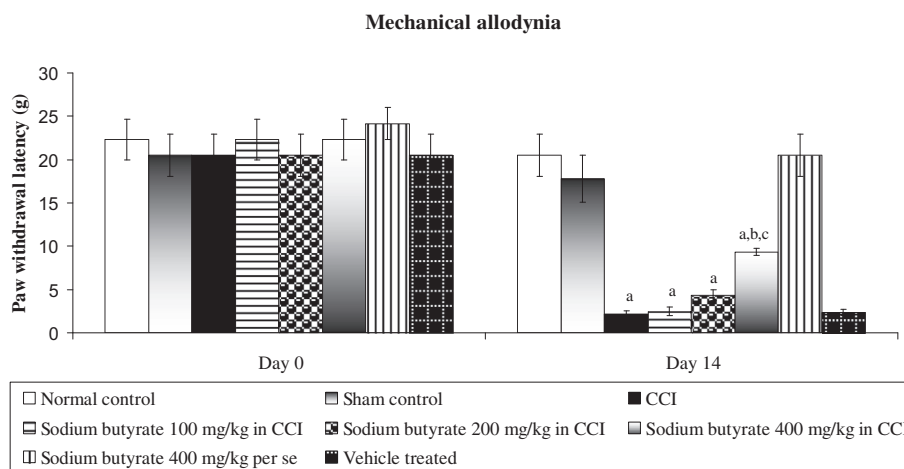


Figure 2 Effect of sodium butyrate on CCI-induced mechanical allodynia assessed by Von Frey hair test. Values are given as mean \pm standard error of the mean, $n = 6$ rats per group. Two-way analysis of variance followed by Bonferonni's *post hoc* test, $F(1, 80) = 137.07$, for days, $p < 0.0001$ and $F(7, 80) = 12.17$ for treatment, $p < 0.0001$. ^a $p < 0.05$ versus sham control corresponding to same day. ^b $p < 0.05$ versus CCI corresponding to same day. ^c $p < 0.05$ versus sodium butyrate 100 mg/kg in CCI corresponding to same day. CCI = chronic constriction injury.

Mechanical hyperalgesia

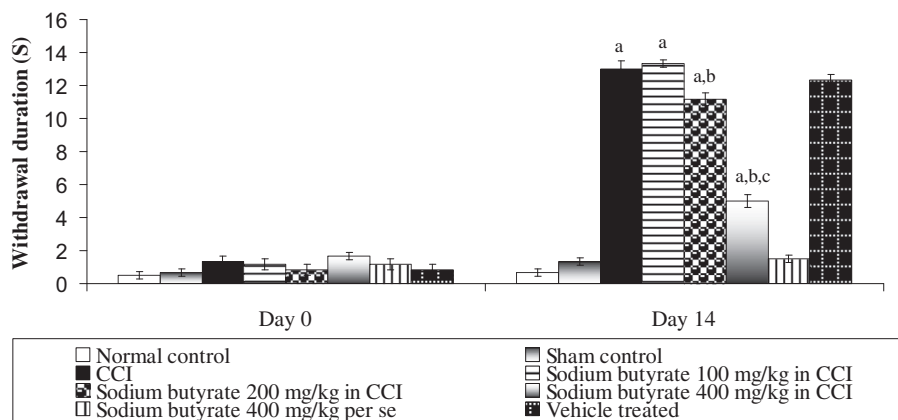


Figure 3 Effect of sodium butyrate on CCI-induced mechanical hyperalgesia assessed by pin prick test. Values are given as mean \pm standard error of the mean, $n = 6$ rats per group. Two-way analysis of variance followed by Bonferonni's *post hoc* test, $F(1, 80) = 1696.65$, for days, $p < 0.0001$ and $F(7, 80) = 182.75$ for treatment, $p < 0.0001$. ^a $p < 0.05$ versus sham control corresponding to same day. ^b $p < 0.05$ versus CCI corresponding to same day. ^c $p < 0.05$ versus sodium butyrate 100 mg/kg in CCI corresponding to same day. CCI = chronic constriction injury.

pain-attenuating effects in neuropathic pain. Sodium butyrate, being a nonselective inhibitor, may also have produced the beneficial effect by inhibiting HDAC I. However, the present study data are not sufficient to differentiate the role of HDAC I and IIa in the pain-attenuating effect that in turn may be established with the use of more selective pharmacological agents.

In the present study, CCI-induced neuropathic pain was associated with an increase in TNF- α level in the sciatic nerve. TNF- α appears early in the cytokine cascade, therefore, it is considered to be a prototype proinflammatory mediator. The role of TNF- α has been well documented in peripheral as well central sensitization in neuropathic pain.³⁰ The results of the present study showing elevated levels of TNF- α in the sciatic

nerve, even on Day 14 in CCI-subjected rats, are consistent with our previous studies^{27,31–33} and those from other laboratories.^{34,35} By contrast, the studies based on neuropathic pain in CCI-subjected mice have demonstrated that the early elevation of TNF- α in the sciatic nerve tends to normalize by Day 3³⁶ or Day 7.³⁷ It is difficult to explain this discrepancy related to early, transient increased expression of TNF- α in a mouse model and prolonged expression of TNF- α in a rat model of CCI. At the most, the possible species difference may have accounted for the different results in these studies. Some studies have suggested the influence of species on TNF- α production, and Foster and co-workers³⁸ have demonstrated that lipopolysaccharide-stimulated TNF- α production is higher in rats than in mice.

Heat hyperalgesia

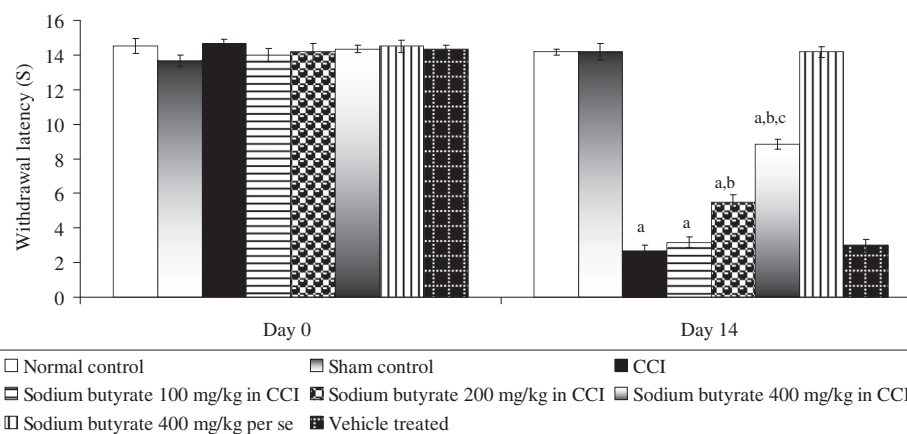


Figure 4 Effect of sodium butyrate on CCI-induced heat hyperalgesia assessed by Eddy's hot plate test. Values are given as mean \pm standard error of the mean, $n = 6$ rats per group. Two-way analysis of variance followed by Bonferonni's *post hoc* test, $F(1, 80) = 1256.39$, for days, $p < 0.0001$ and $F(7, 80) = 118.91$ for treatment, $p < 0.0001$. ^a $p < 0.05$ versus sham control corresponding to same day. ^b $p < 0.05$ versus CCI corresponding to same day. ^c $p < 0.05$ versus sodium butyrate 100 mg/kg in CCI corresponding to same day. CCI = chronic constriction injury.

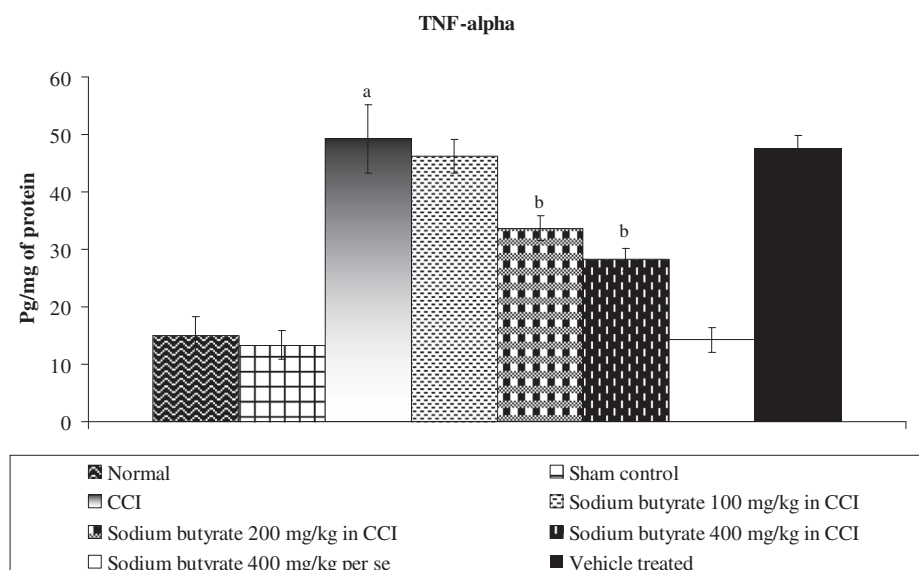


Figure 5 Effect of sodium butyrate on CCI-induced rise in TNF- α level in the sciatic nerve. Values are given as mean \pm standard error of the mean, $n = 6$ rats per group. One-way analysis of variance followed by Tukey's multiple range test, $F(7, 47) = 24.54$, $p < 0.0001$. ^a $p < 0.05$ versus sham control. ^b $p < 0.05$ versus CCI. CCI = chronic constriction injury; TNF = tumor necrosis factor.

The reduction in the levels of TNF- α may be attributed to inhibition of HDAC because some studies have demonstrated that HDAC inhibition reduces cytokine production; particularly cytokines relevant to autoimmune/inflammatory diseases.³⁹ SAHA (suberoylanilide hydroxamic acid) and ITF2357 (Givinostat) (HDAC inhibitors) have been shown to reduce inflammation in animal models of diseases via suppression of TNF- α , interleukin-1 β , interferon- γ , and interleukin-6 production.⁴⁰ The HDAC expression and activity in synovial tissue of rheumatoid arthritis patients correlates positively with the concentration of TNF- α ,⁶ and it has been shown that HDAC inhibitors suppress cytokine production in rheumatoid synovial tissue explants.^{41,42} Sodium butyrate also decreases proinflammatory cytokine expression via inhibition of nuclear factor (NF)- κ B activation and I κ B- α (inhibitor of kappa -light-chain-enhancer of activated B cells) degradation.^{43,44} Place and co-workers have demonstrated that butyrate inhibits NF- κ B activation by HDAC-inhibition-induced suppression of proteasomal activity and stabilizing I κ B- α .⁴⁵ These studies suggest the relationship among HDAC, NF- κ B, and TNF- α . It is possible that the sodium-butyrate-mediated decrease in production of cytokines, including TNF- α , is responsible for its noted beneficial effects in the neuropathic pain in rats.

Recently, Zhang and co-workers⁴⁶ have demonstrated that during persistent inflammatory and neuropathic pain there is histone hypoacetylation, which in turn causes suppression of GABAergic (Gamma amino butyric acid) inhibitory activity, and the effectiveness of HDAC inhibitors in restoring the GABAergic activity in a spinal nerve ligation model. Earlier studies have demonstrated that loss of GABA inhibitory activity in the spinal neurons is associated with neuronal hyperactivation, and restoration of GABA activity is effective in relieving nerve-injury-induced pain.^{47,48} Furthermore, some studies have shown that increased TNF- α levels in neuronal cultures and hippocampal slices decrease GABA inhibitory activity.^{49,50} Based on these results, it may be

hypothesized that the sodium-butyrate-mediated decrease in TNF- α levels and restoration of GABAergic activity are responsible for relieving neuropathic pain in CCI models.

The major limitation of the present work was the lack of direct evidence regarding the involvement of HDAC inhibition in the sodium-butyrate-mediated pain attenuation in CCI-induced neuropathic pain. Therefore, future studies may be directed to study the upregulation of HDAC in neuropathic pain and correlate its upregulation with the generation of proinflammatory cytokines.

In conclusion, sodium butyrate attenuates neuropathic pain manifestations in a CCI model, which may possibly be attributed to its ability to decrease the release of proinflammatory mediators during the nerve injury condition.

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