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Inhibitory effect of valproate sodium on pain behavior in diabetic mice involves suppression of spinal histone deacetylase 1 and inflammatory mediators

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ABSTRACT

Anti-epileptic medications are included in the international guidelines for managing neuropathic pain. Valproate sodium (VPS) was recently described as "the forgotten analgesic" and has been reported to relief pain in various models of neuropathic pain. Some studies reported anti-inflammatory and histone deacetylase 1 (HDA1) inhibitory properties for sodium valproate. The aim of the current study was to investigate the modulatory effect of VPS on pain behavior and inflammatory reactions in alloxan-induced diabetic neuropathy focusing on HDA1 inhibition and glia reactivity. 28 Male Swiss albino mice were allocated into four groups, (1) vehicle group, (2) alloxan-diabetic group, (3 & 4) alloxan + VPS (25 or 50 mg/kg) groups. VPS was given daily for 5 weeks by oral gavage. Pain behavior demonstrated increased allodynia (von-Frey filaments) and hyperalgesia (hot-plate test) in alloxan-diabetic mice that was reduced significantly by at least one of VPS doses. Sciatic nerves in diabetic mice showed increased histopathology score, increased silver staining for the nerves-indicating myelopathy- and a decrease in immunostaining for nerve growth factor. Spinal cord of diabetic mice showed greater histopathologic score, increased CD11b and glia fibrillary acidic protein (GFAP) immunostaining than vehicle treated mice. Molecular investigations highlighted greater content of spinal histone deacetylases, tumor necrosis factor-α (TNF-α) and interlukin-1β (IL1β) that were favorably modified by VPS. Overall, the current data confirmed that the pain killing and anti-inflammatory activity of VPS is at least partly mediated through inhibition of spinal HDA1 and glia reactivity. These findings support the view of inviting antiepileptics for treating neuropathies.

1. Introduction

Diabetic neuropathy (DN) is a common complication among diabetic patients and substantially erodes the patient's quality of life [1] and may lead to amputations and incapacity. It is usually characterized by a gradual progress involving small and large sensory fibers. Symptoms have a glove-and-stocking distribution that start at the lower limbs then progress upwards and include inability to sense pain and evolving of neuropathic pain [2].

Glial cells have emerged as key players of neuropathic pain perception and as targets for drug development. Besides neurons, glia are the other major cell type in nervous system, and comprise diverse, particular cell types within the peripheral nervous system such as Schwann cells, satellite and perineural glia and in CNS such as astrocytes, microglia, oligodendrocytes and perivascular glia [3,4]. Activation of glia occurs upon peripheral nerve damage followed by secretion of a number of proinflammatory factors [5,6].

Several studies have reported that neuropathic pain is linked with

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changes in acetylation and deacetylation of histone proteins, leading to irregular nociceptive gene transcription [7]. Histone deacetylases are phosphoproteins [8] perform a crucial role in epigenetic regulations of pain, either acute or chronic [9–11]. Recent studies have inspected the capability of HDA inhibitors in amelioration of neuropathic pain [12,13] and glia activity in vivo and in vitro [14]. Valproic acid, which interferes with HDA, has appeared to be an effective painkiller under some conditions in rodents [15,16].

Valproic acid and its sodium salt, valproate sodium (VPS), are commonly utilized in treatment of epilepsy and bipolar disorder. Although they are not yet licensed for treating neuropathic pain, both drugs are occasionally prescribed when first-line therapies fail to treat the symptoms. There are some similar biochemical and pathophysiologic mechanisms observed in epilepsy and in neuropathic pain [17]. In the light of this fact, it seems logic that antiepileptic agents can be directed to neuropathic pain treatment [18]. Various rodent models have revealed that VPS possesses neuroprotective and neurotrophic effects. These beneficial effects include suppression of glial cell function [19].

One study dedicated that HDA1 represents a direct target of valproic acid [20]. Here we investigated the effect of repeated VPS treatments in alleviating allodynia and hyperalgesia in a mouse model of DN and the role of spinal HDA1 and glia cell inhibition.

2. Materials and methods

2.1. Chemicals and drugs

Alloxan monohydrate was procured from d fine-chem limited (Mumbai, India) and was dissolved in saline (0.9% NaCl) solution. Valproate sodium was kindly provided by Medical Union Pharmaceuticals (Ismailia, Egypt).

2.2. Housing conditions for the experimental animals

Twenty-eight male Swiss albino mice were provided by Moustafa Rashed Company (Giza, Egypt). Mice had original body weight equals 18–30 g and were kept in polyethylene cages in a normal light/dark cycle with the lights on at 06:00 a.m. Food and water were available ad libitum. Experimental procedures were performed during the light cycle between 11:00 and 15:00 h. Mice were habituated for 8 days before starting the experiments. Experimental procedures were approved with research ethics committee at the Faculty of Pharmacy in Suez Canal University.

2.3. Mouse model of alloxan-induced diabetes mellitus

Animals were overnight fasted with free access to water followed by a baseline measurement of fasting blood glucose (FBG). Induction of diabetes to all groups (except non-diabetic control) was done by an intraperitoneal (i.p.) injection of a standard dose of alloxan (180 mg/kg) dissolved in saline [21]. Diabetic state of mice was confirmed after 120 h following injection of alloxan. Measurement of FBG was done by obtaining a droplet of blood from mice tail vein, placed on a glucose test strip and read using an Accu-Check glucometer. Mice were considered diabetic when FBG levels were above 200 mg/dL [22,23] and are thus enrolled in the study.

2.4. Experimental design

The non-diabetic control group (saline) of 7 mice had received a single i.p. injection of saline followed by administration of distilled water. The rest of mice had induction of diabetes by alloxan single i.p. injection as mentioned above. After confirmation of hyperglycemia, diabetic mice were further subdivided in to 3 groups with 7 mice per each; the first group received distilled water, the second group received VPS [25 mg/kg] and the last group received VPS [50 mg/kg] [24]. Fig. 1 describes the step-wise course of the experiment.

2.5. Mechanical allodynia assay with von-Frey filaments

Mice were adapted to the experimenter before the experiment and acclimatized to the testing apparatus for 30 min before conducting the experiments. Mice were positioned in transparent plastic chambers suspended above a wire mesh grid. Testing was done with a conventional set of nine von-Frey filaments of ascending forces [25]. When the mouse did not show a response, the von-Frey filaments were pushed touching the plantar surface of the paw. A positive response was recorded if the paw was suddenly withdrawn upon application of a filament as well as licking or biting of stimulated paw. Testing began with the smallest filament and progressed according to an up-down method [25]. After a negative reaction, mice were stimulated with the next filament of higher force. Testing of a filament was done for five rounds per paw. The threshold filament size was set as the smaller filament resulted in three withdrawals out of five successive trials [26].

2.6. Thermal hyperalgesia assay using hot-plate test

Lsi LETI-CA hot-plate apparatus (LE 7406 Italy) was utilized for assessment of thermal hyperalgesia. The apparatus includes a transparent cylinder [20-cm in diameter and 25-cm in height] helps to keep the animal on the heated surface. The hot-plate temperature was set at 55 °C using a thermoregulated water-circulated pump. Mice adapted to the experimenter and testing room for 30 min. Each animal was individually placed on the hot-plate and a cut-off time equals 45 s was fixed [27]. The time of latency was recorded which is defined as the period of time between the zero point, when the animal is positioned on the surface of hot-plate, and the time when the animal starts paw licking or successfully jumps off the glass cylinder in order to avoid thermal pain [28,29].

2.7. Histopathology for spinal cord the and sciatic nerve

Scarification of mice was done using the cervical dislocation method under ether anesthesia after the last treatment with VPS and pain behavior assessment. One sciatic nerve and the complete vertebral column were separated and fixed in 4% phosphate-buffered formalin (PBF) for 1 day. Thereafter, the sciatic nerve or the lumbar and cervical parts of the spinal cord were cross sectioned at 4-μm. One section was stained with hematoxylin and eosin (H&E) [30] while another sciatic section was used for silver staining. Sciatic nerves from mice were blindly scored according to nerve fiber degeneration, myelinopathy and axonopathy as follows: 0:absent, 1:mild, 2:moderate, 3:severe and 4:very severe. Similarly, specimens from the spinal cord were also scored blindly as 0:absent, 1:mild, 2:moderate, 3:severe, 4:very severe and 5:extremely severe for the next features: occurrence of eosinophilic foci including degenerated neurons as well as gliosis in the spinal white and the gray matter [31,32].

2.8. Silver staining of sciatic nerves

4-μm cross sections from the sciatic nerve paraffin blocks were subjected to deparaffinization and hydration. Sections were placed in 10% silver nitrate in dark for 30 min at 37 °C, washed and incubated for 15 min at 37 °C in silver nitrate solution added to it concentrated ammonium hydroxide. After that, sections were washed in 0.1% ammonium hydroxide solution. Developer solution was then added (0.2 mL of 37% formaldehyde, 12 mL of distilled water, 12.5 μL of 20% nitric acid and 0.05 g of citric acid) to the silver hydroxide and ammonium hydroxide solutions. Sections were stained in this solution for a period of ten min until a black color develops then washed in 0.1% ammonium hydroxide and toned in 0.2% gold chloride. Sections were fixed in 5% sodium thiosulfate, washed in distilled H_2O, dehydrated in alcohols

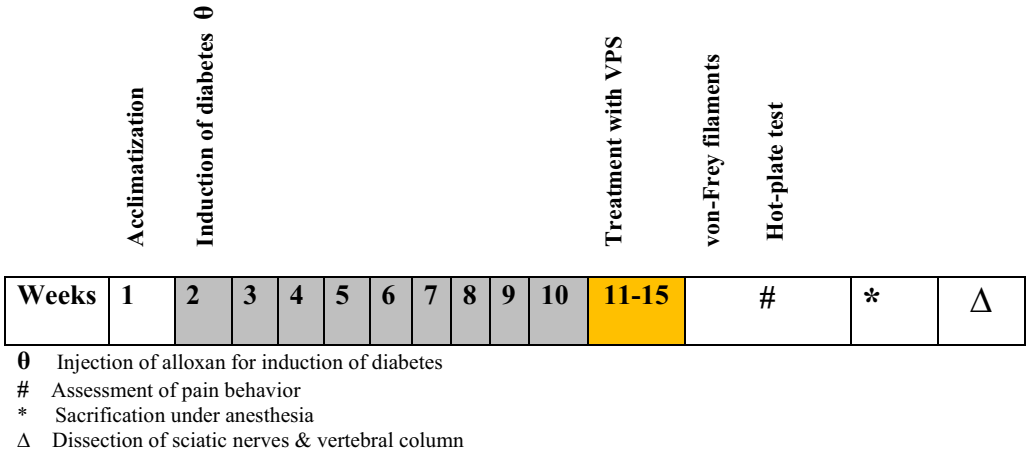


Fig. 1. A diagram illustrating the study design.

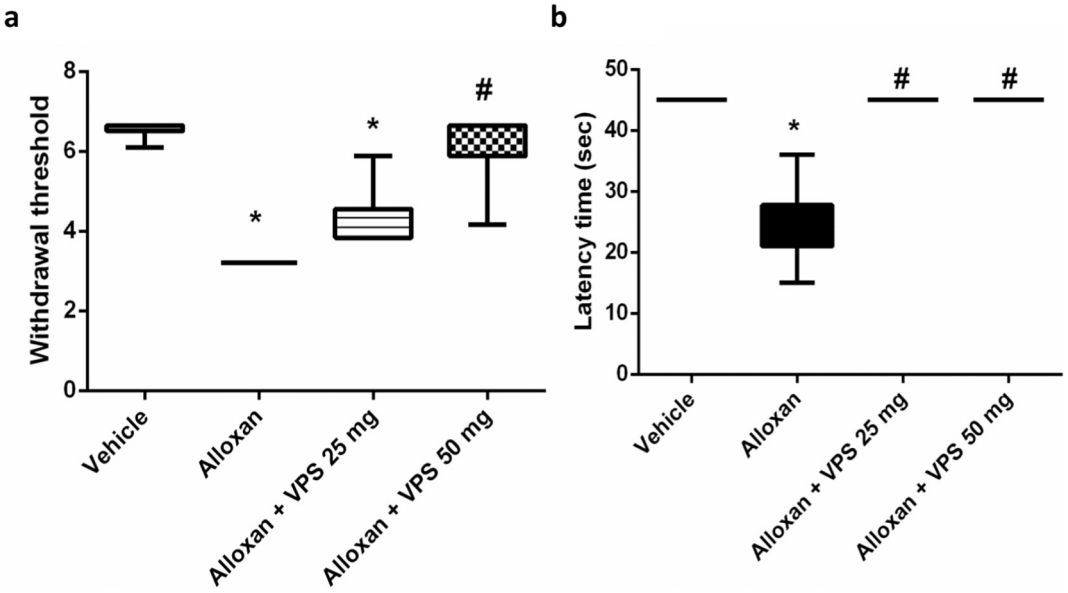


Fig. 2. Effect of sodium valproate on the tactile allodynia and thermal hyperalgesia in alloxan-diabetic mice. A) mean threshold for paw withdrawal, tactile allodynia was measured using a series of von-Frey filaments with ascending order using the up and down methods on the mouse hind paw. B) Latency time in the hot-plate test adjusted at 55 °C and cut-off time equals 45 s. Data are presented as box-Whisker plots and analysis was done using non-parametric ANOVA followed by Dunn's post-hoc test at $P < 0.05$. *versus saline group, # versus the alloxan group.

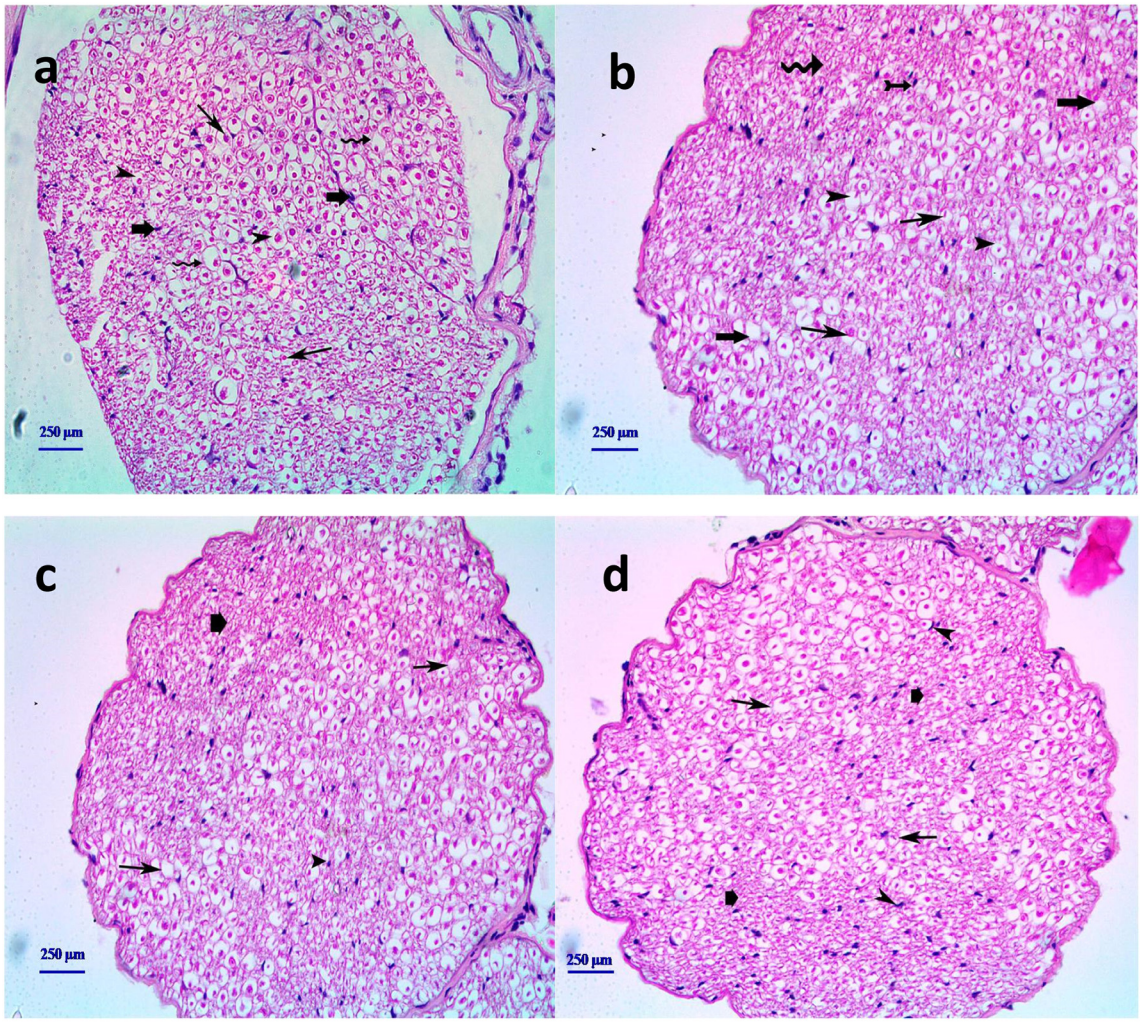
then xylene and mounted [33].

2.9. Immunohistochemistry

Paraffin-embedded sections from the sciatic nerves and cervical spinal were cut at 4-μm thickness and fixed on aminosilane slides. Tissue sections were subjected to deparaffinization and rehydration followed by treatment with 3% H2O2 for a 5-min period for quenching peroxidases. Sciatic sections were stained for NGF using EP1302Y, rabbit monoclonal NGF antibody from GeneTex Inc. (Cat. # GTX61496). A similar protocol was followed to stain the spinal sections by Biorbyt rabbit polyclonal CD11b antibodies (UK, orb11009) or Thermo Fisher Scientific rabbit polyclonal GFAP antibodies (UK, RB-087-A). DakoDenmarkA/s Envision system was applied to complete the staining procedures. Counterstaining for the immunostained sections was done with Mayer's hematoxylin. After that, spinal sections were observed under a light microscope in a blinded manner and image analysis was completed by the ImageJ program (National Institute of Health, Maryland, USA). A number of ten fields representative to the entire section were used for determination of the immuno-reactive area for NGF, GFAP or CD11b.

2.10. Western blot analysis for spinal histone deacetylase enzyme, TNF-α and IL1β

Animals were sacrificed at the indicated time-point (Fig. 1) and samples from cervical spinal cords were swiftly removed and frozen. Mouse spinal cord samples were lysed using modified RIPA buffer that contained proteinase/phosphatase inhibitor cocktail (Thermo Scientific). The tubes were centrifuged for 30 min at 12,000 ×*g* at 4 °C. The supernatants were separated and assayed for total protein content using BCA Protein Assay (Thermo Scientific). Thereafter, equal protein amounts were boiled in Laemmli sample buffer. The denatured samples were loaded on SDS-PAGE gel. Then, the protein was transferred to nitrocellulose membrane followed by incubation of the membrane with antibodies for IL-1β (Abcam, ab9722), HDA1 (Abcam, ab19845) and TNF-α (Abcam, ab6671). The immunoreactivity was detected by horseradish peroxidase-conjugated antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). In addition, membranes were probed for β-actin to confirm equal loading, and the



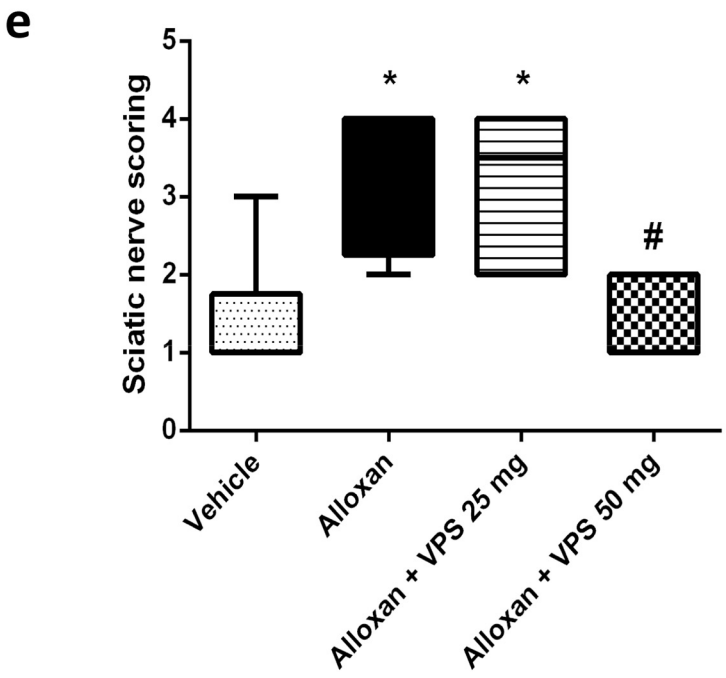


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Fig. 3. Effect of valproate sodium (25 or 50 mg/kg) on the histopathologic picture of the sciatic nerve. Cross sections of sciatic nerves. a) saline group showing multiple axons (arrow) embedded in variable thickness of myelin sheath (arrow head) enclosed within endoneurium (kinked arrow) with multiple nuclei of Schwann cells (thick arrow). b) Alloxan group shows multiple losses of neuronal axons (arrow) with loss of myelin sheath (thick arrow), vacuoles within epineurium (kinked arrow) with some nuclei of Schwann cells (arrow head). c) Mice treated with VPS (25 mg/kg) shows loss of some neuronal axons (arrow) variable loss of myelin sheath (thick arrow) with nuclei of Schwann cells (arrow head). d) Mice treated with VPS (50 mg/kg) shows increased neuronal axons (arrow) variable reduction of myelin sheath (thick arrow) with nuclei of Schwann cells (arrow head) H&E × 400. Scoring was done from 0 to 4 according to the histopathologic findings. e) box-whisker plots representing the median values for the scoring data. Analysis used the Kruskal-Wallis ANOVA followed by Dunn's test at $P < 0.05$. * versus saline group, # versus the alloxan group.

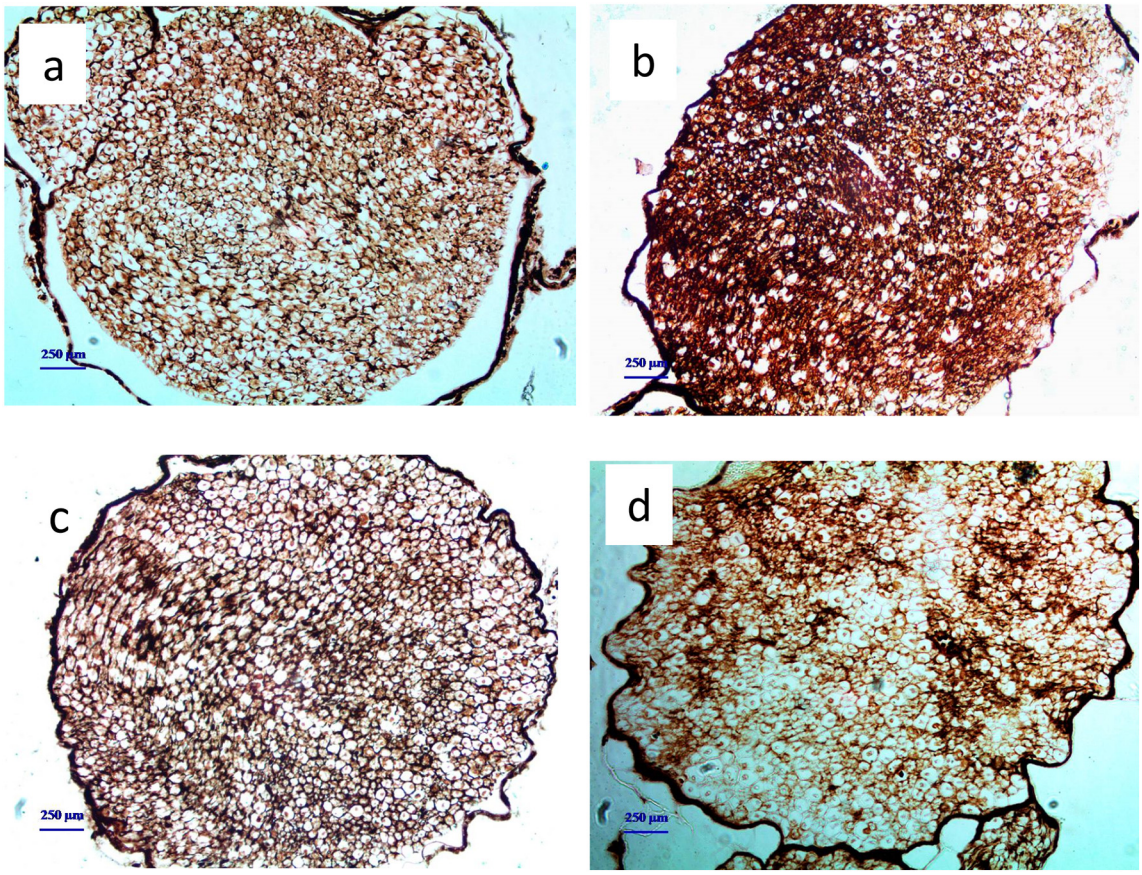


Fig. 4. Silver staining for transverse sections from the sciatic nerve. a) A section from saline group showing well organized axons of nerve fibers with well-developed myelin sheath surrounded by endoneurium, b) A section from alloxan diabetic mice showing considerable reduction in the nerve myelin sheathes and deep staining of silver stain, c) A section from diabetic mice treated with VPS (25 mg/kg)showing: regenerated myelin sheathes and decreased silver stain and d) A section from alloxan-diabetic group treated with VPS (50 mg per kg) showing considerable regeneration of the myelin sheathes and reduction in the silver stain. Silver stain × 400.

Intensity of immunoreactivity was quantified by densitometry.

2.11. Statistical analyses

Statistical analyses were done by applying the GraphPad Prism 6. Parametric data were presented as means ± SE. The significance of differences was analyzed by one-way ANOVA and Tukey's multiple-comparisons test. Non-parametric quantitative data and ordinal data related to histopathologic scoring were presented as medians and quartiles. The significance of differences was analyzed by Kruskal-Wallis ANOVA and Dunn's multiple-comparisons test. P values < 0.05 were considered significant.

3. Results

Measuring the pain behavior revealed that diabetic group revealed smaller threshold value in von-Frey test and shorter latency time in hot-plate test in comparison with the saline group (Fig. 2). Diabetic mice received VPS 50 mg/kg showed significantly bigger withdrawal threshold (Fig. 2a). Regarding hot-plate test, diabetic mice treated with VPS (25 and 50 mg/kg) showed prolonged latency to jump from the apparatus in comparison with the diabetic control mice (Fig. 2b).

Fig. 3 shows specimens from the sciatic nerves stained with H&E. The registered scores in the alloxan diabetic mice were greater than the score of the saline treated mice. Alloxan + VPS (50 mg/kg) group showed lower sciatic degeneration score compared to the alloxan control group (Fig. 3e). Silver staining for sciatic sections revealed that alloxan diabetic mice showed reduction in myelin sheaths and deep silver staining if compared to the normal well-organized axons and myelin sheathes observed in the vehicle group (Fig. 4a–d). Immunostaining for NGF in specimens from sciatic nerves is shown in Fig. 5a–d. Image analysis indicated lower staining area in diabetic mice compared to saline treated mice. Treatment with VPS dose-dependently increased the area of NGF staining (Fig. 5e).

Fig. 6 shows photographs for cervical and lumbar specimens from the spinal cords stained with H&E (I&II). Histopathological examination highlighted greater histologic score for cervical and lumbar sections in the alloxan diabetic mice compared to saline injected mice. The median scores in the alloxan diabetic group and alloxan + VPS (25 mg/kg) group were greater than that obtained in saline group. However,

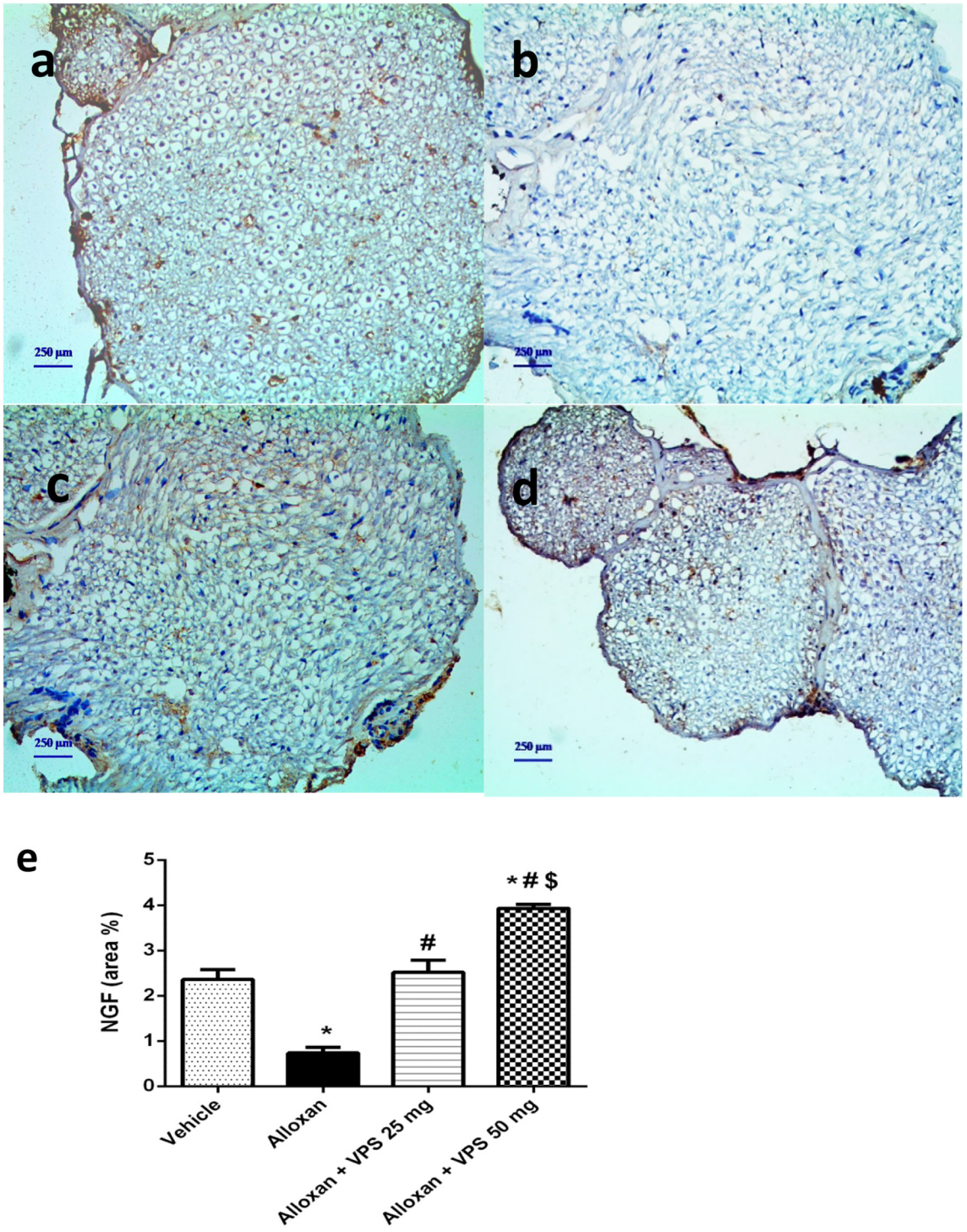
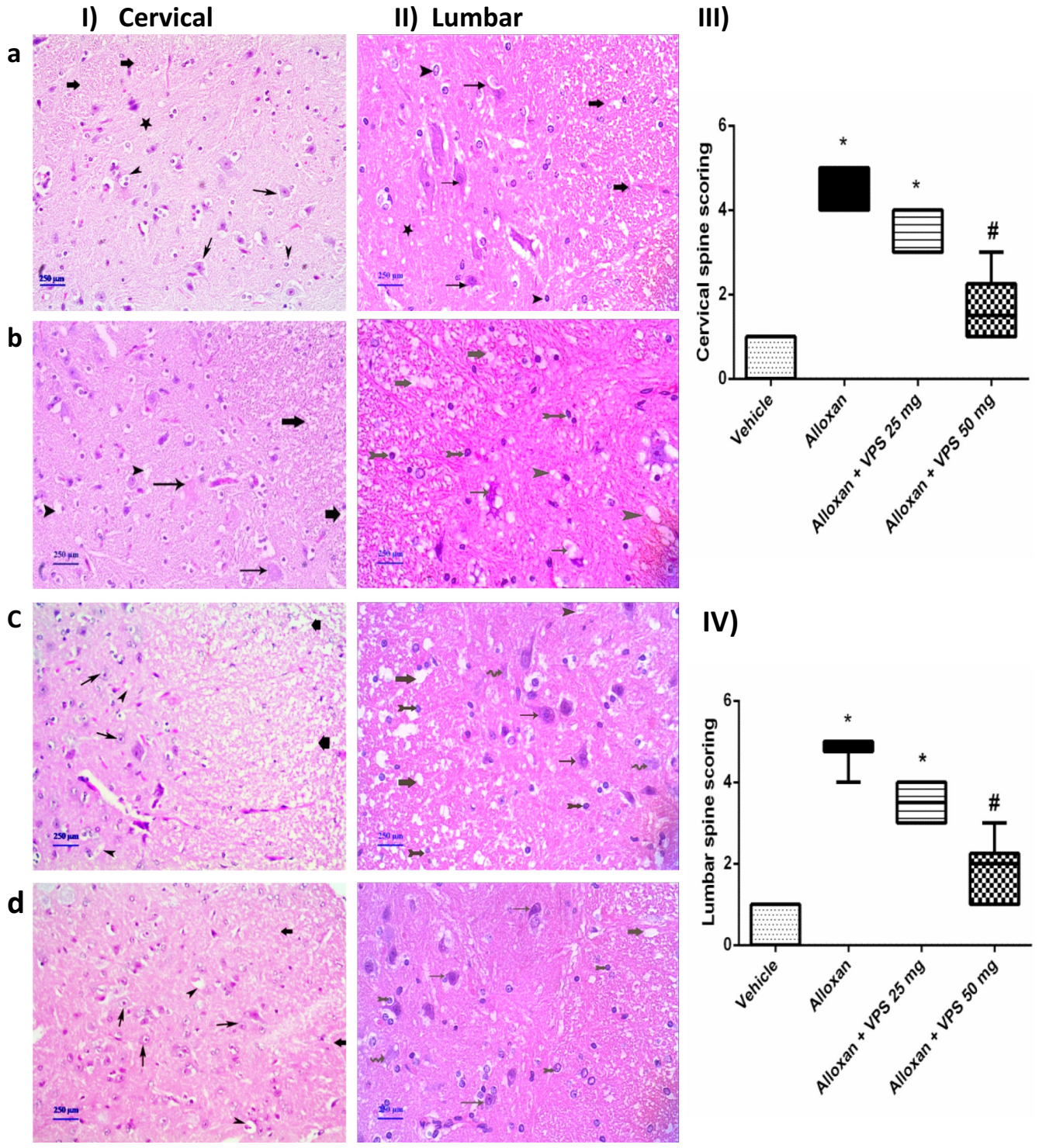


Fig. 5. Immunostaining of sciatic sections for the nerve growth factor. Images for NGF in a) A section from saline group shows basic expression of NGF, b) Alloxan diabetic group shows marked reduction in the expression of NGF, c) Diabetic group cured with VPS 25 mg per kg shows relevant increase in the NGF expression and d) Alloxan-diabetic group cured with VPS 50 mg per kg shows considerable increase in the expression of NGF. NGF immunostaining × 400. e) Column chart demonstrating area of immunostaining for NGF in the experimental groups. Data are mean ± SE, analysis was done by one-way ANOVA and Tukey's test. * versus saline group, # versus the alloxan group, $ versus the alloxan + VPS (25 mg/kg) group.

alloxan + VPS (50 mg/kg) group showed significantly lower median score compared to alloxan diabetic group (Fig 6III&IV).

Fig. 7 shows spinal sections immunostained for GFAP and CD11b (I &II). Data demonstrated greater area (approximately 4-fold and 5-fold) for staining in the spinal cords of alloxan group compared with the vehicle group. VPS (25 or 50 mg/kg) treated mice demonstrated lower % for immunostaining area for GFAP and CD11b in comparison to the alloxan group (Fig. 7III&IV).

Western blot analysis for spinal HAD1 enzyme, TNF-α and IL1β indicated significant increment in alloxan group compared to the vehicle group. Treatment with both doses of VPS (25 or 50 mg/kg) significantly decreased spinal level of these parameters in comparison to the alloxan group (Fig. 8).



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4. Discussion

Studies of pain in humans may be hard to be done and limited from the ethical point of view, thus, rodent models are frequently utilized for studying the pathophysiology of pain. Pain behavior is not easy to be measured in a direct way in rodents and hence, many methods that measure nociceptive behavior have been established by many scientists [34]. In our study, von Frey and hot plate tests are used as stimulus-evoked methods to assess behavioral responses to pain in mice.

The von Frey filaments were created by the scientist Maximilian von Frey. It is used for evaluation of mechanical allodynia in rodents [35]. Our results demonstrated tactile allodynia and thermal hyperalgesia developed in alloxan treated diabetic mice as the threshold for paw withdrawal estimated by von Frey test and latency measured in the hot-plate test were both reduced in this diabetic group. These results were in agreement also to previous reports which indicated that the streptozotocin-diabetic animals show the pathophysiology of peripheral diabetic neuropathy [36,37]. Additionally, abnormal hyper-excitability of nociceptive primary sensory neurons is considered a leading factor toward the exaggerated pain behavior observed in diabetic neuropathy

Fig. 6. Effect of valproate sodium (25 or 50 mg/kg) on the histopathology of the spinal cord. I) sections from the cervical region from a] saline groups showing the gray matter with neural cells (arrow) with prominent nucleus, visible nucleoli and extended axons, glial cell (arrow head), all surrounded by neuropil (star) and white matter packed with axons (thick arrows). b] A section from alloxan diabetic group showing degenerated neural cells (arrow) with nuclear fading, vacuoles in the neuropil (arrow head) with decreased axons and vacuoles in the white matter (thick arrows). c] Mice treated with VPS (25 mg/kg) presented recovered neural cells (arrow) with prominent nuclei, visible nucleoli, extended axons and vacuoles in the neuropil (arrow head) with decreased axons and vacuoles in the white matter (thick arrows). d] A section from mice treated with VPS (50 mg/kg) showing increased recovered neural cells (arrow) with prominent nucleus, visible nucleoli and extended axons while vacuoles in the neuropil are decreased (arrow head) with increased axons and decreased vacuoles in the white matter (thick arrows) H& E × 400. II) Section in the lumber spinal cord presenting a] the saline group shows large sized pyramidal cells (arrow) with multiple glial cells (arrow heads) impeded in neuropil (star) and multiple bundles of white matter are packed with axons (thick arrow). b] A section from alloxan diabetic group showing degenerated pyramidal cells (arrow), multiple haloes within the neuropil (arrow heads) with increased number of glial cells (tailed arrow). Multiple haloes are present within the bundles of white matter axons (thick arrow). c] Mice treated with VPS (25 mg/kg) showing recovered pyramidal cells (arrow), few degenerating pyramidal cells (kinked arrow), few haloes within the neuropil (arrow heads) and increase in the number of glial cells (tailed arrow). Haloes are present within the bundles of the neuronal axons (thick arrow). d] Mice treated with VPS (50 mg/kg) showing increased number of recovered pyramidal cells (arrow), fewer degenerating pyramidal cells (kinked arrow) and decrease in the number of glial cells relative to other groups (tailed arrow). Fewer haloes are present within the bundles of the neuronal axons (thick arrow), H&E × 400. III&IV) box-Whisker plots representing the median values for the scoring data for the cervical and lumbar sections. Scoring was done from 0 to 5 according to the histopathologic findings. Analysis was done using the Kruskal-Wallis ANOVA followed by Dunn's test at $P < 0.05$. *versus saline group, $^{\#}$versus the alloxan group.

[38,39].

In the current study, treatment with the high dose of VPS significantly inhibited allodynia and hyperalgesia in diabetic mice. Similarly, valproic acid was found to reduce paw edema after carrageenan and protected the paw tissues against the inflammatory damage [40]. Sodium valproate is supposed to act via a combination of mechanisms involving membrane stabilization, boosted GABAergic signaling, decreased glutamate excitation at *N*-methyl-D-aspartate receptors and inhibiting serotonergic transmission. VPS inhibits sustained neuronal firing in murine cortical and spinal neurons. An action that thought to be mediated by prolongation of repolarization of voltage-activated Na^+ channels, blocking of T calcium channels and increasing neuronal K^+ conductance [41].

Different pathologies, like nerve injury, are associated with the induction and promotion of chronic pain. They leads to this result by modulating DNA or DNA packaging histones without DNA sequence change. The histones related acetylation regulation appears to be a key player in neuropathic pain which eventually leads to regulation of transcription for various receptors, transporters, and cytokines among many others. Histone acetylation depends on 2 pivotal enzymes: HDAs and histone acetyl transferases. The modulation of either presents great therapeutic potential targeting neuropathic pain [42].

In the current study, spinal gliosis was detected by greater GFAP and CD11b expression as well as high TNF-α and ILlβ. There is considerable evidence that gliosis contributes to clinical pain syndromes. Both microglia and astrocytes are implicated in these phenomena [43]. Historically, the first evidence came from Garrison et al. (1994) who showed an increase of the astrocyte marker GFAP in L4 spinal cord in a neuropathic pain model [44]. Many experimental studies confirmed the development of spinal gliosis and pharmacological disruption of glial activation can produce an anti-nociceptive effect [31,45] and recovers nerve conduction velocity [46] in models of neuropathic pain.

Additionally, diabetes mellitus is characterized by inflammation and increased proinflammatory cytokines production [47]. In the current study, alloxan-diabetic neuropathy led to elevated level of spinal TNF-α and IL1β, indicating an active state of inflammation. Spinal glia activation and inflammation as well as sciatic NGF deficiency were thought as direct reasons in the pathogenesis of hyperalgesia and allodynia encountered in alloxan induced diabetic mice. One review article highlighted the role of immune and glial cell-derived factors as pain mediators and modulators. Perhaps surprisingly, many of the factors control peripheral and central pain processes, although in different ways. The release of key pain mediators including tumor TNF-α and IL1β; these well-known inflammatory cytokines activate and sensitize peripheral nociception and hence contribute to continuing pain [43].

The inflammatory cytokines IL1β and TNF-α when delivered centrally are reported to induce symptoms of neuropathy in rats [48], both are upregulated after peripheral nerve injury [49], and inhibitors of IL1β [50] and TNF-α [51] are capable of reducing nerve injury-induced tactile allodynia. Similarly, IL1β levels were reported to be greater in cerebrospinal fluid subsequent to peripheral inflammation while the block of this rise lessens inflammation-related pain hypersensitivity [52]. There are numerous prospects for cytokine-induced pain modulation. Receptors for IL1β and TNF-α are expressed on spinal neurons or primary sensory neuron terminals. They increase in many persistent pain conditions [53]. Activating these receptors results in quick ameliorations in neuronal excitability [54] or long-term changes in gene expression in neurons that modulate their responsiveness or even to apoptosis [55]. In addition, these cytokines may act indirectly, via the release of PGE2 or NO [56].

Valproate is a powerful inhibitor of HDA enzyme that are proved to play major role in the development of diabetes, controlling the HDA activity is expected to hasten the glucose metabolism and modify the diabetes progression, it also enhances the proliferation and function of pancreatic β cells and remodel microvascular complications [57,58]. In our study, VPS decreased spinal level of HDA, TNF-α. and IL1β; this agrees with a previous report [59]. In addition, VPS stimulated sciatic NGF expression. Taken together, reduced inflammatory markers in addition to increased NGF were supposed to confer an explanation for the ameliorating effects of VPS in reducing the exaggerated nocifensive behavioral responses. Latency period in hot-plate test and withdrawal threshold in von Frey test were both normalized in alloxan treated diabetic mice supplemented with VPS (50 mg/kg), while, VPS (25 mg/kg) increased the latency period in the hot-plate test to the normal value without significantly enhancing the withdrawal threshold. In agreement, a former study had demonstrated that VPS induced a dose-dependent improvement in response to a thermal stimulus or generalized hyperalgesia versus the control rats [15].

Histopathological changes occurring in multiple organs in diabetes may be attributed to the immunological reactions, inflammatory processes and oxidative stress [60–62]. Indeed, neuropathy and neurologic disorders are examples of diabetic complications [63–65]. The chronic state of alternating hyper and hypoglycemia that occur in diabetes affects the cerebrovascular reactivity, neurotransmitters function and metabolism that causes the CNS to be more susceptible to damage as a result of inefficient transport of glucose and ketone bodies [66]. Our study showed improvement in the histopathological changes caused by diabetes in the spinal cord and the sciatic nerve and positive changes in the immunohistochemical markers in response to treatment with VPS especially higher doses, which support the suggestion for VPS treatment of polyneuropathy caused by diabetes [67].

On the other hand, production of cytokines is stimulated by different insults under both and regulate inflammation [68]. The main pro-inflammatory cytokines are TNF-α, IL1β, IL-6 among many others [68]. The presence of these molecules aggravates inflammatory diseases

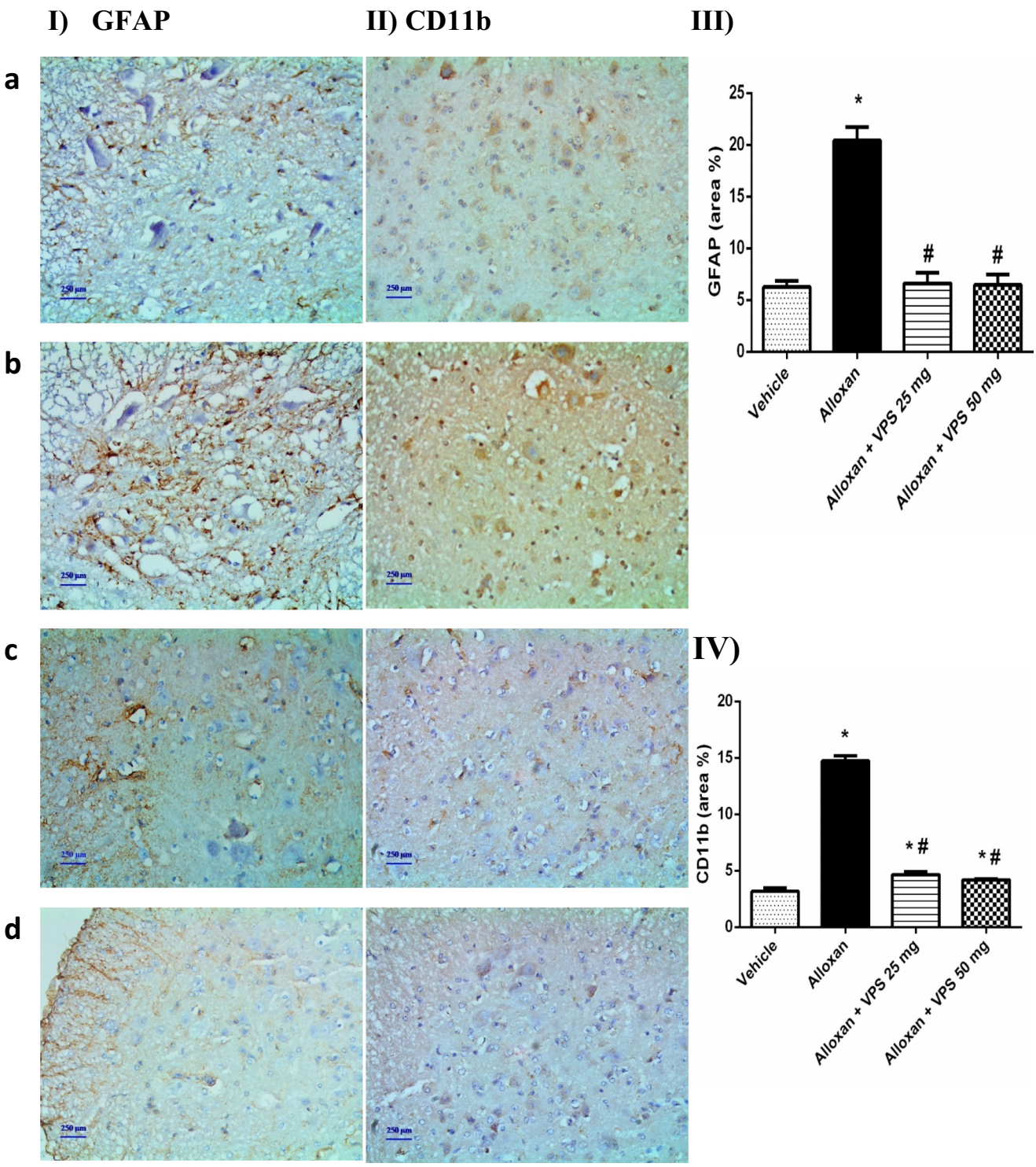

Fig. 7. Immunohistochemical staining for spinal sections in the experimental groups. I&II) Cross sections from cervical sections of the spinal cord stained with GFAP and CD11b immunostaining a) A section from saline group showing basic expression, b) Alloxan diabetic group shows increased expression of GFAP and CD11b in this group, c) Diabetic group cured with VPS 25 mg per kg with decreased expression and d) A section from diabetic group cured with VPS 50 mg per kg with marked reduction in the expression. GFAP and CD11b immunostaining × 400. III&IV) Column charts representing area for immunostaining in the experimental groups. Data are mean ± SE, analysis was performed by one-way ANOVA followed by Tukey's test. *versus saline group, #versus the alloxan group.

[69] and play a vital role in development of neuropathic pain [43]. Earlier studies showed that oral administration of the HDA inhibitor, sodium butyrate, attenuates the increments in TNF-α, and such anti-inflammatory activity triggers the anti-nociceptive activity [70]. Another study showed that paclitaxel induces painful neuropathy in a dose-dependent manner which is concomitant with spinal cord cytokine production such as TNF-α, IL1β and IL-6 [71]. A recent study published by Valvassori et al. showed that HDA inhibition by VPS has led to the downregulation of TNF-α, IL-6 and IL1β in a model of bipolar disorder [6]. The decrease in proinflammatory cytokines has a direct effect on nociception and on the aggravation of diabetic neuropathy [72].

In the present study, the use of VPS modified the pain threshold in

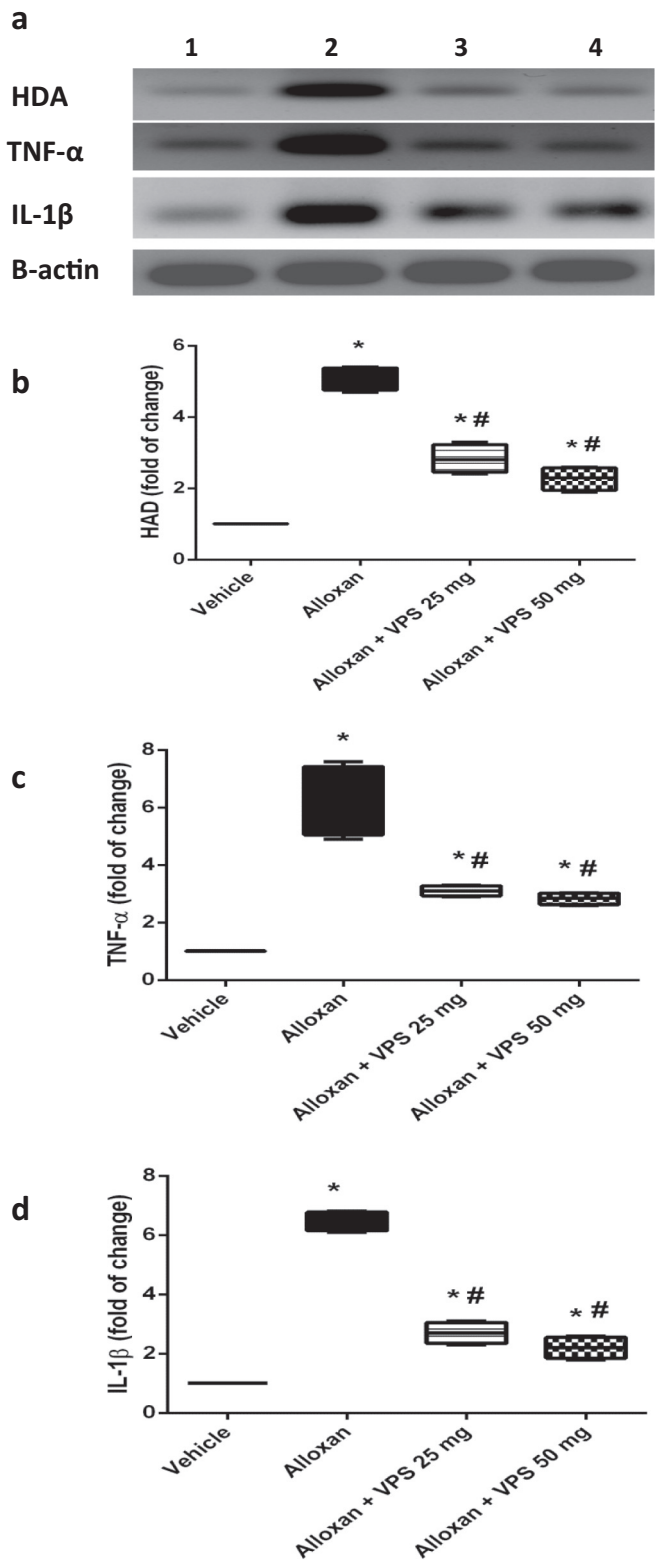

Fig. 8. Western blot analysis for histone deacetylase1, TNF-α and IL-1β in the spinal cord tissue. β-actin was used to confirm equal loading, and the Intensity of immunoreactivity was quantified by densitometry. Column charts representing mean mean ± SE for the measured parameters, analysis was performed using one-way ANOVA and Tukey's test. * versus saline group, # versus the alloxan group.

both the Von Frey filament test and the hot-plate test. It also showed lower sciatic and spinal degeneration. Additionally, it decreased HDA and the proinflammatory cytokines TNF-α and IL1β. Taken together, it seems reasonable to believe that VPS has great potential in the treatment of diabetic neuropathic pain via its HDA and glia inhibiting properties.

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