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Original article

Nrf2 and NF-κB modulation by Plumbagin attenuates functional, behavioural and biochemical deficits in rat model of neuropathic pain

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ABSTRACT

Background: Plumbagin is known to exhibit a broad range of biological activities including anti-cancer, antimicrobial and has been widely used traditionally. Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) inhibitory and Nuclear factor (erythroid derived-2) like-2 (Nrf2) modulatory activities of Plumbagin have been reported already. In nerve injury model of neuropathy in rats, the role of NF-κB upregulation and declined antioxidant defence has been well recognized. So, we evaluated neuroprotective potential of Plumbagin in chronic constriction injury (CCI) of sciatic nerve induced neuropathic pain in male Sprague-Dawley rats.
Methods: Animals were tested for functional, behavioural and biochemical changes. Various markers associated with oxidative stress and inflammatory changes were assessed in the sciatic nerve and dorsal root ganglion (DRG) of the animals exposed to CCI mediated nerve injury.
Results: CCI induced nerve injury led to long-lasting mechanical hyperalgesia, loss of hind limb function and abnormal pain sensation. Plumbagin treatment (10 and 20 mg/kg, *po*) significantly and dose-dependently reversed mechanical hyperalgesia and other functional deficits. There was a marked increase in NF-κB and reduced Nrf2 levels in sciatic nerve and DRG following nerve injury. Plumbagin strengthened the antioxidant defence by improving Nrf2 levels and checked the neuroinflammation by decreasing NF-κB levels in sciatic nerve and DRG.
Conclusions: Together, these results suggested that Plumbagin alleviated CCI-induced neuropathic pain *via* antioxidant and anti-inflammatory mechanisms. Hence, the study suggests that Plumbagin may be useful for the management of trauma-induced neuropathic pain.

Introduction

Neuropathic pain, chronic pain phenotype caused by lesion or damage to the somatosensory nervous system manifested with pathological amplification of pain processing [1]. With an overall prevalence of 7–8% globally, this devastating condition severely affects the quality of life in patients attributed to the cardinal symptoms of neuropathic pain which include hyperalgesia, allodynia [2,3] and spontaneous pain [4]. Injuries to the peripheral or central nervous system can result from chemotherapeutic drugs, hyperglycaemia, ischaemic insults and trauma. Despite of considerable research in this area, there is no effective treatment strategy to significantly combat the altered pain perception in neuropathic pain [5,6]. The underpinning molecular mechanisms involved ectopic activity, impaired inhibitory activity, peripheral and central sensitization are manifested in the form of allodynia, hyperalgesia and spontaneous pain [7,8]. Nerve injury alters the normal physiology of adjacent intact nerve as well as nerves along the nociceptive tract and subsequent neuroinflammation contributed equally for propagation of the nociceptive signals to the spinal horn through dorsal root ganglion [9,10]. It is difficult to manage this chronic pain condition with conventional drugs like tricyclic antidepressants, dual reuptake inhibitors of serotonin and norepinephrine, gabapentin, pregabalin, topical lidocaine and opioid analgesics, *etc.* because of their limited efficacy and serious

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adverse effects [11,12]. Hence, the quest for finding a better therapeutic option for neuropathy and neuropathic pain is still on.

Interestingly, antioxidants of natural origin like resveratrol, verbascoside, *etc.*, have shown potential for the alleviation of neuropathy and neuropathic pain on chronic treatment [13,14]. Hence, we evaluated the effect of Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naturally occurring yellow pigment, in a well established rat model of CCI-induced neuropathic pain which simulates the human peripheral neuropathy [15]. CCI contributes to neuroinflammation and oxidative stress in neurons of DRG and spinal cord through various mechanisms which include, localized inflammation due to ligation of suture [16], altered permeability of blood spinal cord barrier (BSCB) [17], generation of reactive oxygen species [18], invasion of neutrophils [19], macrophages, activation of glial cells and astrocytes [20], *etc.*

Plumbagin has been shown to have antioxidant, anti-inflammatory, antimutagenic, antitumor, antibacterial, cardiotonic, antidiabetic and neuroprotective properties [21–23]. This plethora of biological activities of Plumbagin may be due to its ability to activate cellular defensive pathways like Nuclear factor (erythroid derived-2) like-2 (Nrf2) and to attenuate the pro-inflammatory downstream signalling pathways mediated by Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) [22]. A recent screen of activity of Plumbagin and its derivatives has clearly demonstrated their potential in neuroprotection under cerebral ischaemia through activation of Nrf2 pathway [24].

Various published reports documented the critical role of oxidative stress and neuroinflammation in pathogenesis of peripheral nerve injury [25,26]. This alters the expression of several chemical mediators primarily in the injured nerve and passes to the spinal cord through DRG causing persistent pain [27,28]. Nrf2 and NF-κB regulation coordinates the redox status of a healthy cell but under neuronal injury, this homeostasis gets perturbed leading to exaggerated oxidative stress and neuroinflammation [29,30]. Therefore, activation of endogenous Nrf2 helps in scavenging the reactive oxygen and nitrogen species by hemoxygenase-1 (HO-1) and glutathione (GSH) [31]. Simultaneous abrogation of NF-κB activation and inhibition of its related proinflammatory cytokines release is hypothesized to protect the neurons against the inflammatory damage and cell death. Hence, the current study was aimed to evaluate the neuroprotective potential of Plumbagin in nerve injury induced neuropathic pain using a rat model.

Materials and methods

Experimental animals

Male Sprague-Dawley rats weighing 180–220 g were group housed and habituated to the housing environment (12 h light/dark cycle, lights on at 7:00 AM) for at least 3 days before the initiation of behavioural studies. Experimental protocols were approved by the Institutional Animal Ethics Committee. Animals were maintained in accordance with the animal guidelines laid by The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All pain tests were conducted in similar time of the day, in accordance with the recommendations of the International Association for the Study of Pain and efforts were made to minimize animal suffering. Basal readings were obtained before CCI and the treatment effect was screened on 7th and 14th day. The dose was administered daily in the morning at the same time. After 2 h of administration the behavioural tests were performed on 7th and 14th day. Person evaluating functional and behavioural studies was blinded for the group assignment to avoid bias. The animals were double labelled and then final data was back analyzed with the original groups of the animals.

Drugs and chemicals

All the chemicals including Plumbagin and Tween 80 were procured from (Sigma, USA) unless specified. Tissue protein extraction reagent (T-PER) was purchased from Thermo Scientific, USA. All the antibodies were purchased from Santa Cruz Biotechnology, USA and Cell Signaling Technology, Beverly, MA, USA. All the chemicals and solvents used were of analytical grade. Isoflurane was obtained from Raman and Weil Pvt. Ltd. (Mumbai, India).

Induction of CCI and drug treatment

Peripheral neuropathy in rats was induced by CCI of sciatic nerve initially developed by Bennett and Xie [15]. Briefly, rats were anaesthetized under isoflurane during surgery. The left sciatic nerve was surgically exposed and four loosely tied ligatures (4.0 chromic gut) were placed around the nerve (1 mm apart) proximal to the trifurcation to prevent the interruption of blood circulation through the epineurial vasculature. For sham group animals, sciatic nerve was exposed but no ligature was applied. After surgery, the muscle and skin was closed through standard procedure and the animals were allowed to recover. Rats were randomly assigned to 5 groups each contains 8 animals and they include normal control, sham control, model control, two Plumbagin treated groups. The two distinct schemes of treatment have been adopted *i.e.* 10 and 20 mg/kg, *po* and administered as suspension with 20% Tween-80 for 14 days (total 14 doses were given per animal; 10 mg/kg and 20 mg/kg doses were fixed based on literature support [21,32,33]). During the study, no mortality was observed. After 14 days of treatment, rats were euthanized with CO_2 and the ipsilateral sciatic nerve; dorsal root ganglia (DRG) were collected and stored at −80 °C to conduct further experimentations.

Functional tests

Assessment of sciatic functional index (SFI)

SFI was calculated by following a method described by Bian et al. [34]. Animal hind paws were dipped in Indian ink and allowed to walk on a white paper placed in an enclosed walking track. Then the following measurements were taken from the footprints of the rat, (i) Print length (PL), (ii) Toe spread (TS) and (iii) Intermediary toe spread (ITS). All the three measurements were taken from both ipsilateral (I) and contralateral (C) sides. Several footprints were obtained from each track and average values of three-foot prints were considered. From these values, various factors were calculated as follows to obtain the sciatic functional index (SFI). (i) Print length factor (PLF) = (IPL-CPL)/CPL; (ii) Toe spread factor (TSF) = (ITS − CTS)/CTS; (iii) Intermediary toe spread factor (ITF) = (IIT − CIT)/CIT. SFI was calculated by using the Bain–Mackinnon–Hunter (BMH) formula; $SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITF - 8.8$. An SFI value for the normal foot is 0 and total impairment is 100, which indicates complete transection of the sciatic nerve.

Behavioural tests

Assessment of thermal hyperalgesia

Animals were habituated to the experimental conditions prior to the experiment. Hot and cold plate tests were used to assess thermal hyperalgesia at (52 ± 1 °C) and (4 ± 1 °C) with a cut-off time of 15 s and 60 s respectively [35].

Assessment of mechanical allodynia

Before the experimentation, all animals were acclimatized in transparent perspex boxes on a mesh surface and Von Frey filaments (Samitek, USA; 1, 2, 4, 6, 8, 10 and 15 g) [36] were applied