Lycopene and ketotifen potentiates the anti-hyperalgesic effect of gabapentin in PSNL-induced neuropathic pain in wistar rats
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Abstract: The present study is designed to investigate the involvement of antioxidant mechanism and role of mast cells in pathophysiology of neuropathic pain induced by partial sciatic nerve ligation. The effect was evaluated by assessing various behavioural parameters (thermal hyperalgesia, cold hyperalgesia), biochemical parameters (lipid peroxidation, reduced glutathione, superoxide dismutase and catalase). Partial Sciatic nerve ligation (PSNL) significantly caused thermal hyperalgesia, cold hyperalgesia and oxidative damage as compared to normal and sham group. Upon daily administration of combination of lycopene (50 mg/kg), ketotifen (10mg/kg) and gabapentin (100 mg/kg) considerably reversed hyperalgesia, cold hyperalgesia and also attenuated oxidative stress when compared to PSNL group. The results indicated that combination lycopene, ketotifen and gabapentin exhibited a potentiating effect in neuropathic pain by inhibiting degranulation of mast cells and also by reducing oxidative stress.

Keywords: lycopene, ketotifen, gabapentin, degranulation, hyperalgesia

Introduction
Neuropathic pain is the most excruciating of all the pains. The current pharmacological treatment of neuropathic pain affords insignificant relief and for that reason it is major healthcare concern. It is estimated that 3% of the population suffers from it and about 25% of patients with chronic pain will have neuropathic pain. Neuropathic pain can be stimulus evoked, spontaneous, or can be a combination of both. Neuropathic pain is difficult to treat mainly because of its resistance to medications and adverse effects associated with medication. At present the pharmacotherapy of neuropathic pain includes anticonvulsants, antidepressants, opioidal and non opioidal analgesics [1, 2]. Neuropathic pain is a severe and unbearable condition which affects approximately 4 million people in the India alone [3]. Patients with neuropathic pain often report sensory abnormalities including paresthesias (numbness or tingling), dysesthesias (electric shock phenomenon), hyperesthesia (increased sensitivity to mild painful stimuli), hyperalgesia (increased sensitivity to normally painful stimuli), hyperpathia (pain produced by subthreshold stimuli), spontaneous pain and allodynia (pain produced by normally non-painful stimuli) [4]. The diverse etiologic causes of neuropathic pain include infectious agents, trauma, metabolic diseases, neurodegenerative diseases, ischemia and drugs [1]. Various studies show that inflammatory events initiated by nerve injury have a fundamental role in the pathogenesis of neuropathic pain. These primarily include inflammatory mediators such as mast cells, nitric oxide (NO) macrophages, cytokines, toll-like receptors, schwann cells, satellite glial cells, microglia, astrocytes, interleukins, nerve growth factor and tumor necrosis factor-α (TNF-α). Involvement of mast cells and oxidative damage has also been reported in the pathophysiology of neuropathic pain and its related complications [5, 6] but antioxidants have been well-known to scavenge reactive oxygen species (ROS) and are increasingly being considered as shielding agents in the management of a various human diseases. The highly lipophilic carotenoid lycopene is the most potent antioxidant present naturally in many fruits and vegetables. Various studies have shown that lycopene inhibits the release of TNF-α and NO [7]. It has also been reported that mast cell degranulation at the site of injury releases histamine which is known to interact with pain and inflammation mainly through H1 [8,9] and H4 [10,11]. Therefore, it can be deducted that mast cell stabilizers and antioxidant could possibly inhibit pain and inflammation [12,13]. This research was aimed to evaluate the effect of lycopene to quench the free radicals produced as a result of the increased oxidative stress and the ability of ketotifen to prevent histaminergic nociception when administered with gabapentin in neuropathic pain induced by partial sciatic nerve ligation (PSNL) in wistar rats [14,15].

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Materials and Methods

Animals: All experiments were performed on adult male wistar rats weighing 150-300 g. The animals were procured from the Animal House, L.T.S Paramedical College (Pharmacy) Muradnagar, Ghaziabad. Animals were housed in groups of 8 per polypropylene cage, maintained at 23±2°C; 55±5% humidity in a natural light and dark cycle. Rats were given ad libitum access to standard food pellets and water. The experiments were performed during the light cycle in awake, freely moving animals that were adjusted to laboratory conditions before proceeding with the experiments.

Induction of peripheral neuropathy: Partial sciatic nerve ligation (PSNL/Seltzer model) was used to induce peripheral neuropathy. This model has been developed by Seltzer et al., [16] and is one of the most commonly used models of neuropathy. The rats were anesthetized with Ketamine (50 mg/kg, I.P.) and Xylazine (5 mg/kg, I.P.). The right hind legs were shaved, and the skin was sterilized with iodine. All surgical instruments were sterilized before surgery. The right hind leg of rat is dissection is made to expose the sciatic nerve at the upper-thigh level. The dorsal one-third to half of the sciatic nerve is tightly ligated with an 8-0 silk suture just distal to the point at which posterior biceps semitendinosus nerve branches off (figure 1). Behavioral estimations were done on 7th and 14th day. At the end of study (i.e. on 14th day) the rats were euthanized for collection of nerve tissue for biochemical estimations [17].

Drug Treatment schedule: The animals were divided into five groups of eight rats. First, second and third group were treated as naïve (vehicle treated), sham group (exposure of the sciatic nerve but not ligated) and control (sciatic nerve ligated animals) respectively. Group four was administered Gabapentin100 mg/kg and fifth group was administered combination of Lycopene 50 mg/kg, Ketotifen 10 mg/kg and Gabapentin 100 mg/kg. In this experiment all the groups of eight rats each were administered drugs once daily orally for the duration of 14 days. All the groups have undergone behavioural and biochemical tests. Doses of gabapentin, Lycopene and ketotifen were selected based on reported literature [18,19].

Behavioral Examinations

a) Hot plate test Thermal hyperalgesia was assessed by placing each animal on hot plate (Eddy’s Hot Plate) which was maintained at 55 °C with a cut off time of 15 sec maintained throughout the experimental procedure. This was carried out on 7th day after induction of neuropathic pain and on the 14th day before animal sacrifice. The latency to first sign of paw licking or jumping response to avoid thermal pain was taken as the index of pain threshold [20].

b) Cold hyperalgesia Cold hyperalgesia was assessed by measuring paw (both ipsilateral and contralateral) withdrawal latency (PWL), when dipped in water bath which was maintained at 4°C ± 2°C on 7th and 14th day after PSNL [21]. A cut off time of 15 sec was maintained throughout the experimental protocol [22].

Biochemical estimations

a) Dissection and homogenization: Behavioural assessments were done on the 14th day and after that animals were sacrificed by spinal dislocation. The complete sciatic nerve was removed and 10% (w/v-1) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). Homogenate was centrifuged for 20 minutes at 15000 rpm and the supernatant was used for estimation of lipid peroxidation and reduced glutathione levels. The post nuclear fractions for catalase assay are obtained by cold centrifugation of the homogenate at 1000 × g for 20 min, at 4°C and for other enzyme assays centrifuged at 12,000 × g for 60 min at 4°C.

b) Lipid peroxidation assay- The lipid peroxidation was performed according to the method of Ohkawa et al [23]. The quantitative estimation of malondialdehyde (MDA) was calculated by reaction with (TBA) thiobarbituric acid 535 nm using Shimadzu Spectrophotometer. The values were calculated using molar extinction coefficient of chromophore (1.56×10⁶/m/ cm/1) and expressed as n moles formed per mg of protein in the tissue [24].

c) Protein Estimation

The protein content was calculated according to the method of Lowry et al., using bovine serum albumin as standard. Protein reacts with the folin’s ciocalteau phenol reagent to develop a colored complex. The color developed is due to reaction of alkaline copper with the protein and the reduction of phosphomolybate by tyrosine and tryptophan present in the protein [25].

d) Estimation of reduced glutathione Reduced glutathione concentration was anticipated according to the method illustrated by Ellman [26]. 1 ml supernatant was precipitated with 1ml of 4% sulfosalicylic acid and cold digested at 4°C for 1h. The sample was then cold centrifuged at 1200 × g for 15 min at 4°C to 1ml of this supernatant, 2.7 ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The yellow color produced was immediately measured at 412 nm using Shimadzu Spectrophotometer (uv-1800). Results were calculated using molar extinction coefficient of chromophore (1.36 × 10⁴ M-1cm-1) and expressed as percentage moles (μ moles) per gram of tissue weight.
c) Superoxide Dismutase (SOD) was estimated according to the method defined by Marklund and Marklund [27]. The supernatant was assayed for SOD activity by subsequent the inhibition of pyrogallol autoxidation. 100 micro liters (μl) of cytosolic supernatant was added to Tris HCL buffer. At least 25μl of pyrogallol was added and a change in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

f) Catalase (CAT) A 10% tissue homogenate was prepared in 2.0 ml of phosphate buffer. This homogenate was centrifuged at 3000 rpm for 15 min. Catalase activity was measured in supernatant obtained after centrifugation. 2.95 ml of 19mM hydrogen peroxide was put in cuvette. To it, 50 μl of cytosolic supernatant was added and changes in absorbance at 240 nm were recorded at one-minute interval for three minutes. Presence of catalase decomposes hydrogen peroxide leading to a decrease in absorbance [28].

Statistical Analysis:
All the results are expressed as mean ± standard error mean (SEM) followed by analysis of variance (ANOVA) along with Dunnett’s test using statistical package for the social sciences (SPSS) software. The p<0.05 was considered to be statistically significant.

Results
Effect on Cold hyperalgesia: Cold hyperalgesia was assessed by measuring paw (both ipsilateral and collateral) withdrawal latency (PWL), when dipped in water bath maintained at 40°C ± 2°C on 7th & 14th day after PSNL. A cut off time of 15 sec was maintained throughout the experiment procedure. The PWL was evaluated on 7th day as well as on 14th day. Normal control group was found to be significant when compared to the PSNL group. Combination of Lycopene (50 mg/kg), Ketotifen (10 mg/kg) and Gabapentin (100 mg/kg) significantly (P<0.01) increased the paw withdrawal latency time as compared to PSNL group (group 3) and gabapentin alone treated group (group 4) on 7th (fig. 2) and 14th day (fig. 3).

Figure 1: Partial Sciatic Nerve Ligation (PSL/Seltzer model)

Figure 2 (Cold hyperalgesia 7th day): All values were expressed as Mean ± S.E.M. (n=8); *= P<0.01 when compared with PSNL; †= P<0.01 when compared to Gabapentin 100 mg/kg. (ANOVA followed by Dunnett’s test).

Figure 3 (Cold hyperalgesia 14th day): All values were expressed as Mean ± S.E.M. (n=8); *= P<0.01 when compared with PSNL; †= P<0.01 when compared to Gabapentin 100 mg/kg. (ANOVA followed by Dunnett’s test).

Figure 4 (Hot Plate 7th day): All values were expressed as Mean ± S.E.M. (n=8); *= P<0.01 when compared with PSNL; †= P<0.01 when compared to Gabapentin 100 mg/kg. (ANOVA followed by Dunnett’s test).

Figure 5 (Hot Plate 14th day): All values were expressed as Mean ± S.E.M. (n=8); *= P<0.01 when compared with PSNL; †= P<0.01 when compared to Gabapentin 100 mg/kg. (ANOVA followed by Dunnett’s test).
Effect on Hot Plate: Thermal hyperalgesia was measured by placing each animal on a hot plate (Eddy's Hot Plate) maintained at 55°C throughout the experimental procedure. Thermal hyperalgesia was assessed on weekly intervals on 7th (fig. 4) & 14th (fig. 5) day after PSNL. The latency to first sign of paw licking or jumping response to avoid thermal pain was taken as an index of pain threshold. A cut off time of 15 sec was retained during the experiment [29]. Normal control group was found significant when compared to PSNL group. While combination of Lycopene (50 mg/kg), Ketotifen (10 mg/kg) and Gabapentin (100 mg/kg) significantly (P<0.01) increased the jumping time when compared against PSNL group (group 3) and gabapentin alone treated group (group 4) (Figure 3 & 4).

Effects of Lycopene, Ketotifen and gabapentin on oxidative damage: Sciatric nerve ligation significantly caused oxidative damage as indicated by increased lipid peroxidation, depletion of reduced glutathione level, superoxide dismutase and catalase activity in sciatic nerve. Treatment with Lycopene (50 mg/kg), Ketotifen (10 mg/kg), Gabapentin (100 mg/kg) significantly (P<0.01) reverse the oxidative stress by decreasing the lipid peroxidation (fig. 6) and increasing the reduced glutathione (fig. 7), SOD (fig. 8) and catalase (fig. 9) level when compared to PSNL group (group 3) and gabapentin alone treated group (group 4) on 14th day.

Discussion

Pain is an obnoxious sensory and emotional experience that may have a considerable impact on quality of life, general health, psychological health, and social and economic wellbeing of the patient. The International Association for the Study of Pain defines neuropathic pain as “pain caused by a lesion or disease of the somatosensory nervous system. Central neuropathic pain is defined as ‘pain caused by a lesion or disease of the central somatosensory nervous system”’. The characteristic descriptions of the neuropathic pain include sensory symptom such as shooting, stabbing, like an electric shock, burning, tingling, tight, numb, prickling, itching and a sensation of pins and needles. Patient may describe symptoms of allodynia (pain caused by a stimulus that does not normally provoke pain), hyperalgesia (an increased response to a stimulus that is normally painful), anaesthesia dolorosa (pain felt in an anaesthetic [numb] area or region), and sensory gain or loss (IASP 2011) [30]. It is well known that mast cells infiltrate at the site of inflammation and get degranulated at the site of injury and release mediators such as histamine, serotonin, proteases, prostaglandins and cytokines. Several mast cell mediators have the ability to sensitize nociceptors, including histamine [7,8] and tumour necrosis...
factor-\(\alpha\) (TNF-\(\alpha\)) [31] resulting in increased firing rates & activation of nociceptors. Few researches also indicate the increased free radicals at the site of injury which further damage the neuronal membranes. Lycopene and Ketotifen both have ability to neutralize ROS. Additionally, Ketotifen also has H1 antihistaminic action and non-specific anti-inflammatory action. Management of neuropathic pain is very tricky because of heterogeneity of its aetiologies, symptoms and complex underlying mechanisms [32]. Thus, there arises a need to explore novel pharmacological treatments for neuropathic pain. Consequently, this study aimed out to assess and authenticate the effects of mast cell stabilizer ketotifen and antioxidant Lycopene in neuropathic pain and also to understand the involvement of mast cells and ROS in pathophysiology neuropathic pain. The results of the present study clearly showed that treatment with gabapentin (100 mg/kg) significantly attenuated sciatic nerve ligation induced behavioral alterations in pain perception. Combined treatment with ketotifen (10 mg/kg), Lycopene (50 mg/kg) and gabapentin (100 mg/kg) was found to be more effective in alleviating the symptoms of neuropathic pain and significantly increased the paw withdrawal latency time in hot plate and cold hyperalgesia assessment. There have been suggestions that antioxidants have the ability to reverse the peripheral nerve injuries. Out of various suggested pathways for nerve injury antioxidant and lipid peroxidation have been major focus as the lipids are the chief constituent of nerve cell membranes and peripheral nervous system. Therefore, lipid peroxidation is potentially damaging as it affects the permeability of neuronal membranes and further affects the composition and integration of surface receptors and enzymes. Ketotifen (10 mg/kg), Lycopene (50 mg/kg) in combination with gabapentin (100 mg/kg) reduced the levels of lipid peroxidation and restored the low levels of glutathione, catalase and superoxide dismutase suggesting that the combination of ketotifen and gabapentin can be employed in treatment of neuropathic pain. Hence, it may be concluded that ketotifen exerts ameliorative effect in neuropathic pain possibly by inhibiting degranulation of mast cells and reducing oxidative stress by increasing the nitric oxide synthase activity [33].

The results of the present study evidently demonstrate the involvement of mast cell degranulation and antioxidant mechanism. The results also indicate the potentiating effect ketotifen (10 mg/kg) and lycopene (50 mg/kg) when administered with gabapentin (100 mg/kg) against the neuropathic pain induced by PSNL.

Conclusion

Based on the results obtained above the present study suggests that administration of combination lycopene, ketotifen and gabapentin exhibited a potentiating therapeutic effect on rats with experimentally induced neuropathic pain due to high antioxidant and free radical scavenging action of Lycopene and ketotifen as well ability of ketotifen to regulate inflammatory mediators involved in the pathophysiology of neuropathic pain. Nevertheless, a better understanding on the detailed mechanism would be helpful and thus necessitate further investigation.

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