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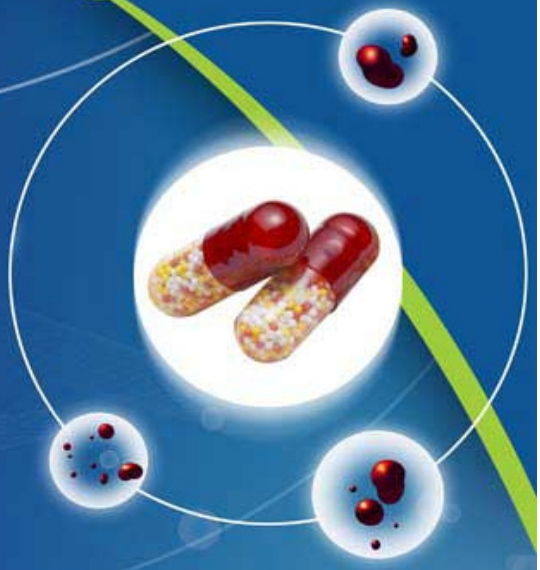

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Research Article

EFFECT OF ZONISAMIDE ON CHRONIC CONSTRICTION INJURY INDUCED NEUROPATHIC PAIN IN MALE SD RATS

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ABSTRACT

There is considerably research evidence supporting a palliative role for voltage-gated sodium and T-type calcium channels in neuropathic pain conditions. Hence, the present study was undertaken to assess the ability of zonisamide (a sodium and T-type calcium channel blocker) to relieve various symptoms of neuropathic pain in the chronic constriction injury rat model of neuropathic pain. Zonisamide (80 & 50 mg/kg) or saline was administered in a blinded, randomized manner by intraperitoneal injection from postoperative day (POD) 7 to 13. Paw withdrawal duration (PWD) to spontaneous pain, chemical allodynia and mechanical hyperalgesia, and paw withdrawal latency (PWL) to mechanical allodynia and thermal hyperalgesia were tested before surgery, before and after zonisamide or saline administration (from POD7 to 13) and after the withdrawal of treatment (from POD14 to 36). Systemic zonisamide relived neuropathic pain symptoms in a dose dependent manner. All PWDs were significantly decreased and PWLs were significantly increased after zonisamide administration compared with saline control measurements. However, zonisamide had non-uniform effect on chemical allodynia. Results of zonisamide were also compared with standard drug pregabalin (50 & 30 mg/kg) and found that zonisamide should be considered as an ulternative pharmacological tool for treatment of neuropathic pain that is largely refractory to standard analgesics such as pregabalin.

Key words:*Neuropathic pain, Allodynia, Hyperalgesia, T-type Ca^{2+} channel, Zonisanmide, Pregabalin*

INTRODUCTION

Neuropathic pain is defined as 'pain initiated or caused by a primary lesion, injury or dysfunction in the central or peripheral nervous system' and is an area of largely unmet therapeutic need [1].

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Patients with neuropathic pain frequently demonstrate spontaneous pain, thermal and mechanical hyperalgesia and allodynia [2]. There is an increasingly large body of evidence suggesting a role for voltage-gated Na^+ and Ca^{2+} channels in pain pathologies [3-8]. Voltage-gated sodium channels (VGSCs) are critical elements of action-potential initiation and propagation. Deregulation of VGSCs expression is thought to be involved in changes to neuronal firing and contribute to neuropathic pain states [4], while more

recently discovered T-type Ca^{2+} channels become activated after a small depolarization of the neuronal membrane and therefore play a crucial role in excitability of both central and peripheral neurons [5-7]. Recent study demonstrated that T-type currents are up-regulated in a chronic constriction injury induced animal model (CCI model) of peripheral neuropathy and thus play important role in development of neuropathic pain following peripheral nerve injury [8]. It has been reported that microglia, the resident macrophages and principal immune response cell in the CNS, are massively activated in the dorsal horn soon after peripheral nerve injury and release many immune modulators including nitric oxide that contribute to the induction and maintenance of neuropathic pain by altering neuronal function [9-11]. At present, there are very few effective and well-tolerated therapies for neuropathic pain. Zonisamide, which has been developed as a new generation antiepileptic drug is expected to be clinically effective for treatment of this chronic disease [12, 13]. Multiple modes of action have been reported for zonisamide, including inhibition of sodium channels and T-type calcium channels [14, 15], scavenging of free radicals [16], and blockade of nitric oxide (NO) synthesis [17]. In the light of these observations, the objective of this study was to determine whether parenteral zonisamide relieves neuropathic pain in murine CCI model.

MATERIAL AND METHODS

Animals and maintenance

Male Sprague-dawley rats of body weight between 200-230 gm were used for neuropathic pain model. All experiments were approved by the Institutional Animal Ethics Committee (1622/PO/a/12/CPCSEA). Each rat was housed in plastic box cage individually with well controlled supplied air, humidity (‹ 70%) and temperature under a 12 hour light/dark cycle with food and water *ad libitum*.

Drugs and chemicals

Pregabalin was obtained from Torrent Research Centre (Gandhinagar, Gujarat) and used as positive control. Zonisamide was obtained from Glenmark Pharmaceuticals (Mumbai, Maharastra). Both drug were dissolved in isotonic saline solution to prepare drug solutions of specific concentration.

Induction of peripheral mononeuropathy (CCI model)

Unilateral mononeuropathy was produced in rats using the CCI model essentially as described by Bennett and Xie [18]. The rats were anesthetized with intraperitoneal combination of Ketamine and xyalazine at 60 and 6.5 mg/kg respectively. The left hind leg was shaved, moistened with a disinfectant, and then common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic trifurcation, about 7mm of the nerve was freed of adhering tissue, and four loose ligatures were made with 4.0 braided silk suture with about 1 mm spacing. The wound was then closed by suturing the muscle using chromic catgut with a continuous suture pattern. Finally the skin was closed with 3.0 black braided silk sutures using a horizontal-mattress suture pattern. Sham surgery was performed by exposing the sciatic nerve as described above, but not damaging it. Povidone iodine ointment was applied topically to the wound and benzyl penicillin antibiotic (20,000 IU/Kg, b.i.d) was given intramuscularly for 5 days after surgery. The animals were then transferred to their home cage and left to recover.

Sensory testing (Nociceptive assay)

Five nociceptive assays aimed at determining the severity of behavioral neuropathic parameters, namely spontaneous pain, allodynia and hyperalgesia, were performed. The assays involved measurement of the degree of spontaneous (ongoing) pain and tests of hind-limb withdrawal to cold, thermal and mechanical stimuli (dynamic mechanical

allodynia, cold allodynia, mechanical hyperalgesia and thermal hyperalgesia). Separate group of animals (n=4) was used for each assay.

Spontaneous pain

Spontaneous pain was assessed for a total time period of 5 min as described previously by Choi et al., [19]. The operated rats were individually placed inside an observation cage and an initial acclimatization period of 10 min was given to each of the rat. Consider of noting the cumulative duration that the rat was holds its ipsilateral paw off the floor. The paw lifts associated with locomotion or body repositioning was not count. For each measurement three successive readings were taken without any elapse and the mean was calculated.

Dynamic component of mechanical allodynia

Dynamic allodynic response was assessed according to the procedure described by Field et al., [20]. The operated rat was placed inside an observation cage. An initial acclimatization period of 10 min was given to each of the rat. A positive dynamic allodynia response consisted of lifting the affected paw for a finite period of time in response to mild stroking on the planter surface using a cotton bud. This stimulus is non-noxious to a normally behaving rat. The latency to paw withdrawal was then noted. If no paw withdrawal was shown within 15 sec, the test was terminated and it was assigned as 'no response'. For each measurement three successive readings were taken with 3 min elapsed between each test and mean was calculated.

Cold allodynia

The rats demonstrating unilateral mononeuropathy were assessed for acute cold allodynia sensitivity using the acetone drop application technique, as described by Caudle et al., [21]. The operated rat was placed inside an observation cage and allowed to acclimatize for 10 min. A few drops (100-200µl) of freshly dispend acetone were squirted as a fine mist on the midplanter region of the affected paw. A cold allodynic response was assessed by noting the duration of the paw withdrawal response. For each measurement, the paw was sampled three times with 3 min interval between each test and mean was calculated.

Mechanical hyperalgesia

Mononeuropathic rats were assessed for mechanical hyperalgesia sensitivity according to the procedure described by Gonzalez et al., [22]. The operated rat was placed inside an observation cage and allowed to acclimatize for 10 min. Hind paw withdrawal duration was measured after a mild pin-prick stimulus to the midplanter surface of the ipsitateral hindpaw. A withdrawal was defined as being abnormally prolonged if it is lasted at least 2 sec or more. The mean duration of withdrawal was taken from a set of three responses with 3 min elapsed between each test.

Thermal hyperalgesia

Thermal hyperalgesia response was assessed according to the procedure described by Eddy and Leimbach [23]. The temperature of eddy's hot plate was set at 55.0 ± 0.1°C. The operated rats was placed on the heated surface, and the time interval between placement and the shaking, licking, or tucking of the affected hindpaws was recorded as the latency response. If no paw withdrawal was shown within 22 sec, the test was terminated and it was assigned as 'no response'. For each measurement three successive readings were taken with 3 min elapsed between each test and mean was calculated.

Drug administration

Baseline sensory response were measured for each group of animals (n=4) preoperatively and 36 days postoperatively according to predetermined manner. Animals showing all five neuropathic pain parameters in baseline measurement (0hr) on post-operative day7 (POD7) were then administered the relevant drug by intraperitoneal route according to predetermined randomized table till POD13 and tests performed again at 1hr, 2hr and 3hr

after drug administration along with baseline measurement (0hr). After day 13, only base line measurements were taken till POD36. Each group of animals was used for only one drug administration and for one parameter to ensure no 'carry-over' effects. Zonisamide (80 and 50 mg/kg) was administered at t = 0 after baseline measurement. Two positive control groups were run alongside drug treatment using pregabalin (50 and 30 mg/kg, respectively). A vehicle control group was also run using saline. The treatment protocol remained the same for these five groups. No drug testing was performed for sham-operated rats.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined for drug effects by one-way ANOVA followed by *Bonferroni post-hoc* test for multiple comparisons. Comparison results with $p < 0.05$ were considered statistically significant. The statistical software package SPSS (version 17.0) was used for the analysis.

RESULTS

All animals included in this study exhibited characteristics neuropathic pain behavior in base line measurement (0hr) on post-operative day7 (POD7) after CCI surgery when compared with preoperative values except sham-operated animals.

spontaneous pain: PWD

Administration of zonisamide after baseline measurement on POD7, completely reversed the spontaneous pain response at both doses (80 and 50 mg/kg), after 1hr of drug administration (4.0*±1.22 s and 2.5*±0.29 s respectively vs. 44.25±8.74 s for control, *$p <$ 0.05) and the effect continuously maintained till POD13, except on 3hr of POD7 and 0hr of POD11 in case of lower dose (Fig.1). Standard drug pregabalin showed protection at both doses (50 and 30 mg/kg) from 1hr of POD7 (4.5*±0.65 s and 15.5*±3.12 s respectively vs. 44.25±8.74 s for control, *$p < 0.05$) and effect continuously maintained during whole treatment period. Pregabalin at dose 50 mg/kg was observed to be about 9 times more effective than zonisamide, in baseline measurement on POD9. On withdrawal of treatment on post-operative day 13, zonisamide (80 mg/kg) and both doses of pregabalin were showed effect only on POD14, while zonisamide at dose 50 mg /kg was effective till POD20 (4.75*±1.11 s vs. 14.25±3.71 s for control, *$p < 0.05$).

Mechanical allodynia: PWL

Administration of zonisamide after baseline measurement on POD7 reversed the allodynic response at dose 80 mg/kg, after 2hr of administration (14.0*±0.41 s vs. 8.5±0.65 s for control, *$p < 0.05$) and on continuous dosing the effect maintained till POD13, except on 0&1hr of POD9 and 0hr of POD13 (Fig.2). Zonisamide was ineffective in reversing the allodynic response at dose 50 mg/kg during whole treatment period, except on 1&2hr of POD13. Standard drug pregabalin showed continuous protection at dose 30 mg/kg, from 3hr of POD9 (11.75*±0.85 s vs. 7.5±0.65 s for control, *$p < 0.05$) to POD13, except at 3hr of POD11 and at 0hr of POD13, while at dose 50 mg/kg, effective only on 3hr of POD7 and 1hr of POD13 (11.5*±1.19 s at 3hr of POD7 vs. 4.5±0.29 s and 13.0*±0.41 s at 1hr of POD13 vs. 6.5±0.65 s for control respectively, *$p <$ 0.05). Zonisamide and pregabalin were devoid of any antiallodynic effect after withdrawal of treatment, except zonisamide at 80 mg/kg reversed mechanical allodynia on POD36 (9.0*±0.71 s vs. 5.25±0.48 s for control, *$p <$ 0.05).

Chemical allodynia: PWD

Zonisamide at both doses gave non-uniform trend of effects during whole study period (PODs 7- 36) (Fig.3). Zonisamide at dose 80 mg/kg was observed to be more effective than 50 mg/kg in measurement on 2hr of POD7 and 2&3hr of POD9. Standard drug pregabalin at dose 30 mg/kg showed complete protection till POD13 after administration on POD7, while at dose 50 mg/kg gave continuous effect till

POD9. After withdrawal of treatment, none of the drugs showed positive effects.

Mechanical hyperalgesia: PWD

Hyperalgesia evoked by a mechanical pin-prick stimulus was effectively attenuated till 2hr of POD13 by zonisamide at dose 50 mg/kg, while dose 80 mg/kg showed non-uniform trend of effects in measurement during whole treatment period (PODs7-13) (Fig.4). Standard drug pregabalin at dose 50 mg/kg was observed to be much effective and showed continuous effect than 30 mg/kg during whole treatment duration. Pregabalin at dose 50 mg/kg was observed to be 4 times more effective than 30 mg/kg, in measurement on 2hr of POD11 (1.5*±0.29 s for 50 mg/kg vs. 6.0±0.71 s for 30 mg/kg, *p < 0.05). On withdrawal of treatment on POD13, zonisamide and pregabalin were devoid of any positive effect on mechanical hyperalgesia, except pregabalin 50 mg/kg on POD14.

Thermal hyperalgesia: PWL

Both doses of zonisamide (80 & 50 mg/kg) significantly improved paw withdrawal latency after one hour of administration on POD7 (18.75*±0.75 s for 80 mg/kg and 18.25*±0.85 s for 50 mg/kg vs. 10.75±1.11 s for control, *p < 0.05) and the effect was continuously maintained till POD13, except in baseline observation on POD9,11&13 (Fig.5). Zonisamide at dose 50 mg/kg, was observed to be more effective than 80 mg/kg, at 3hr on POD9 (20.25*±0.25 s for 50 mg/kg vs. 15.5±0.65 s for 80 mg/kg, *p < 0.05). Both doses of standard drug pregabalin showed complete protection from 2hr of administration on POD7 (19.5*±0.65 s for 50 mg/kg and 18.75*±0.63 s for 30 mg/kg vs. 11.0±0.91 s for control, *p < 0.05) to POD13, except at 30 mg/kg dose in baseline measurements on POD9, 11 and 13. On withdrawal of treatment, continuous post-treatment effect was not observed in any case.

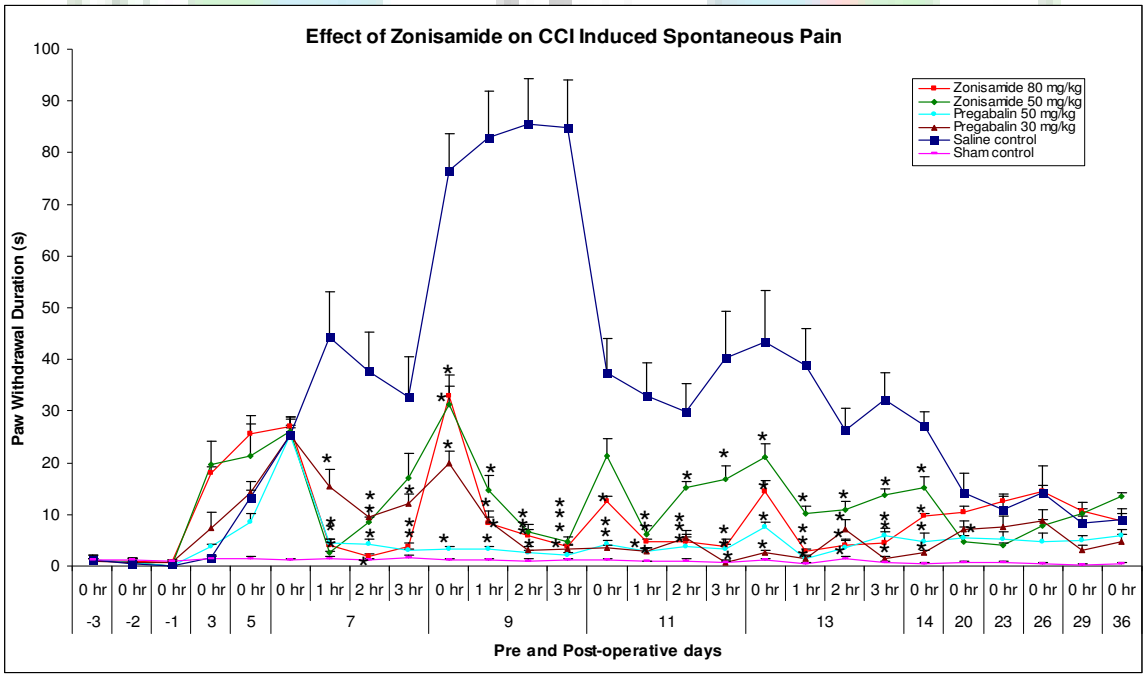

Fig.1- Effect of zonisamide in reversing the spontaneous pain response in CCI rats along with pregabalin. The results are shown as the mean paw withdrawal duration (mean PWD±SEM) of 4 rats per group. *p < 0.05, in comparison with control values (one-way ANOVA followed by *Bonferroni post-hoc* test).

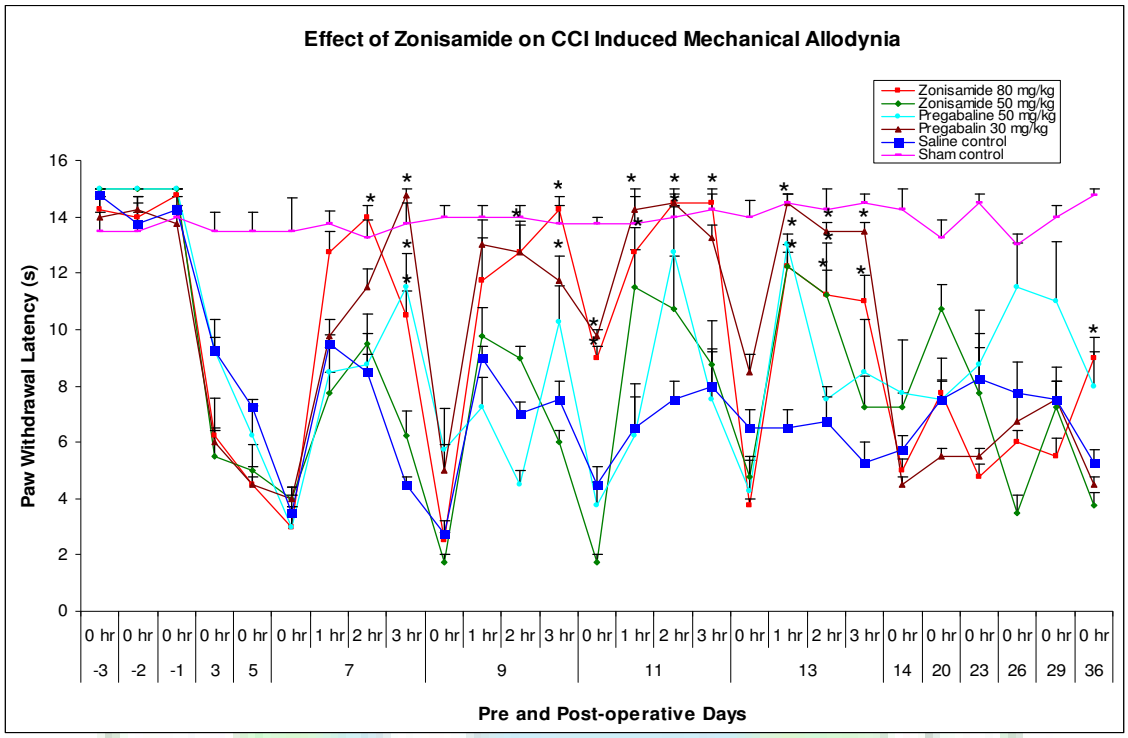

Fig.2- Effect of zonisamide in reversing the mechanical allodynia response in CCI rats along with pregabalin. The results are shown as the mean paw withdrawal latency (mean PWL±SEM) of 4 rats per group. *p < 0.05, in comparison with control values (one-way ANOVA followed by *Bonferroni post-hoc* test).

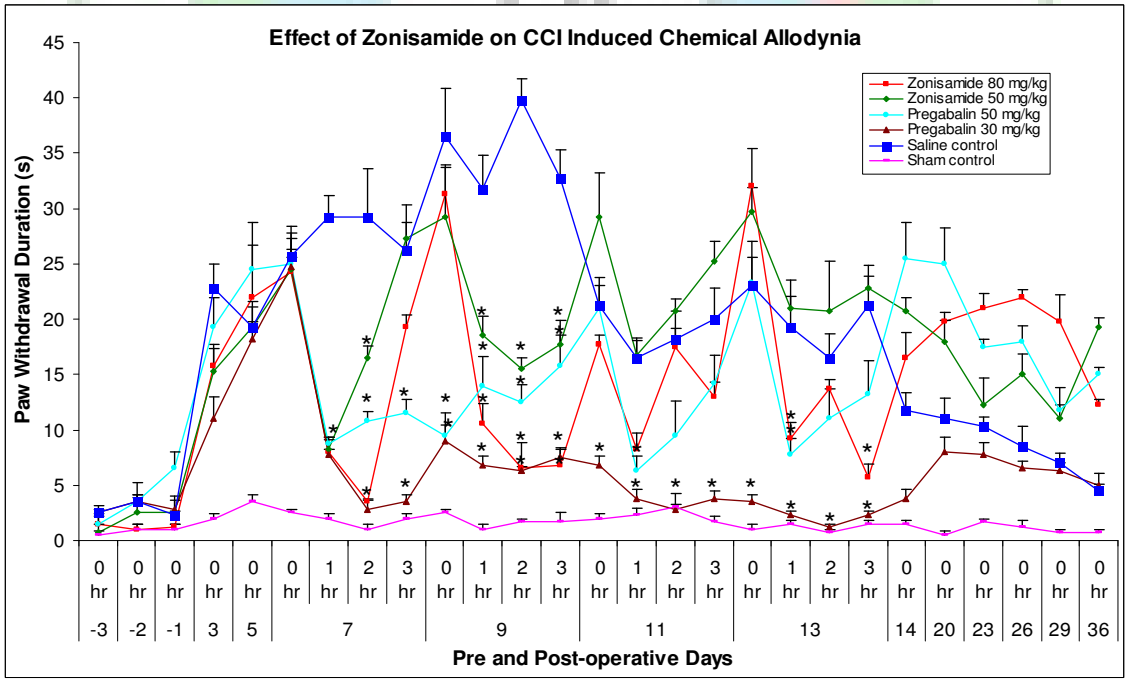

Fig.3- Effect of zonisamide in reversing the chemical allodynia response in CCI rats along with pregabalin. The results are shown as the mean paw withdrawal duration (mean PWD±SEM) of 4 rats per group. *p < 0.05, in comparison with control values (one-way ANOVA followed by *Bonferroni post-hoc* test).

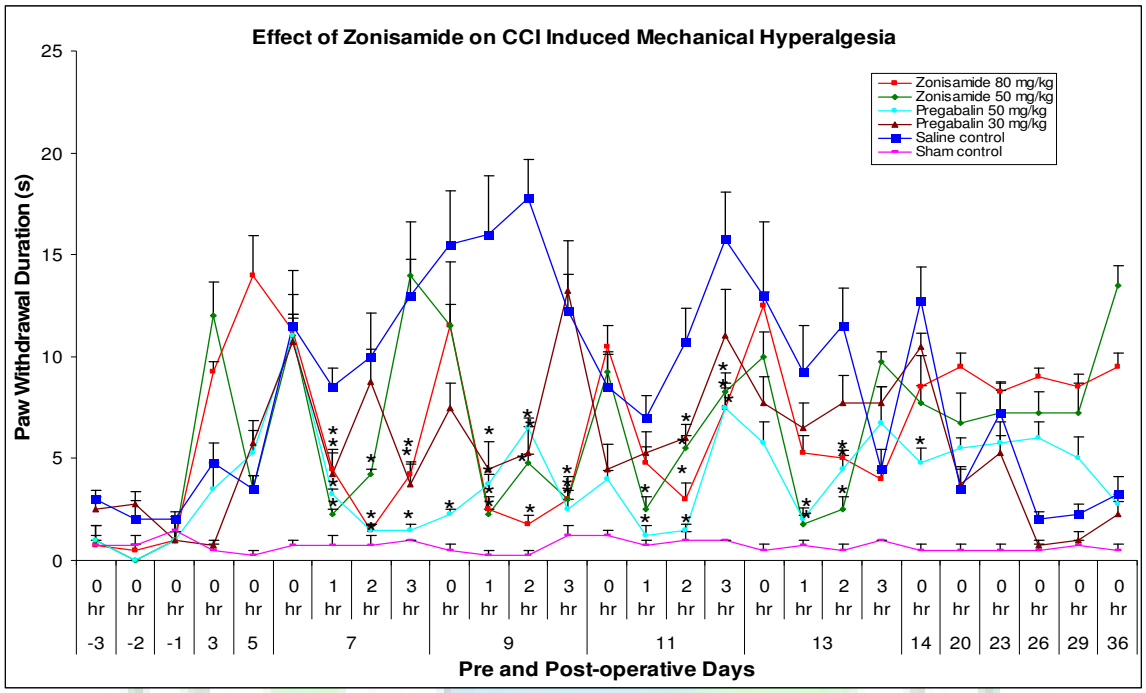

Fig.4- Effect of zonisamide in reversing the mechanical hyperalgesia response in CCI rats along with pregabalin. The results are shown as the mean paw withdrawal duration (mean PWD±SEM) of 4 rats per group. *p < 0.05, in comparison with control values (one-way ANOVA followed by *Bonferroni post-hoc* test).

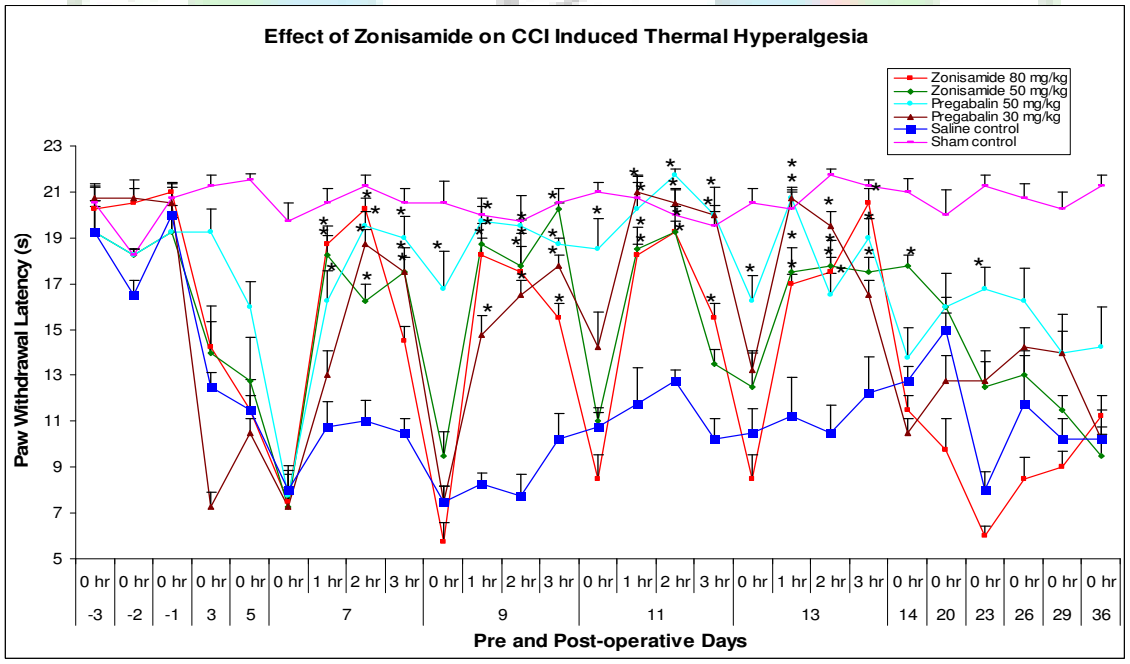

Fig.5- Effect of zonisamide in reversing the thermal hyperalgesia response in CCI rats along with pregabalin. The results are shown as the mean paw withdrawal latency (mean PWL±SEM) of 4 rats per group. *p < 0.05, in comparison with control values (one-way ANOVA followed by *Bonferroni post-hoc* test).

DISCUSSION

This study examined the potential therapeutic value of Na^+ and T-type calcium channel blocker 'zonisamide' in the treatment of neuropathic pain using the CCI model of experimental neuropathy. Zonisamide inhibits neuronal voltage-dependent sodium and T-type calcium channels, both of which have a pivotal role in membrane excitability. Neuronal injury produced by CCI, results in changes in sodium channel numbers and types, which contribute to the hyperexcitability [4, 24-26]. T-type calcium channels may regulate the rate of repetitive neuronal firing, further contributing to the hyperexcitability [5-8, 27]. Blockade of sodium channels suppresses sodium-dependent action potentials, and inhibition of T-type calcium channels attenuates the sharp depolarization of the membrane potential underlying sodium dependent action potentials [28]. Zonisamide also inhibits the synthesis and release of the nitric oxide from activated microglial cells after peripheral nerve injury, thus prevents the alteration in neuronal function [9-11, 17].

In the current experiment, zonisamide caused a dose-related reduction in the spontaneous pain induced by CCI and had a sustained effect at the 80 mg/kg dose during the whole treatment period but at 50 mg/kg dose effect was seen only after 7 days of final drug administration. The possible mechanism behind this may be the increase spontaneous discharge after nerve injury result of phenotypic changes in the nature and distribution of sodium and calcium channels, which occur throughout the damaged neurons, including the dorsal root ganglion. These changes may result not only in spontaneous pain but also contribute to central sensitization [29]. These results are important because zonisamide is the first drug tested in our laboratory that had such a prolonged effect. The reason for this prolonged carryover effect is not clear, but may be related to the long half-life of the zonisamide in rats. Standard drug pregabalin showed positive effect at both doses and the effect was maintained till last dose but no carry over effect was observed.

The mechanism of allodynia (mechanical and chemical) is complex and is not completely understood. Histological changes in peripheral nerve and dorsal root ganglion, spinal cord and supraspinal nerve are accompanied by functional changes, such as peripheral and central sensitization and sympathetic excitation, resulting in increased hyperexcitability of the central nervous system and activation of nociceptive neurons by non-noxious stimuli [30]. Nerve injury induced mechanical allodynia probably involves abnormal discharge originating in nerve injury by activation of Na^+ channels, leading to increased spontaneous and evoked neural activity [4], along with sprouting of Aβ-fibers into the superficial dorsal horn and synaptic rearrangement with central sensitization [2]. The mechanisms underlying nerve injury induced chemical allodynia have been less well documented. The facilitated response of C-fibers or membrane property changes in Aδ cells might be associated with chemical allodynia [31, 32]. Central sensitization mechanisms might also be involved in chemical allodynia [2]. Thus, the inhibition of mechanical and chemical allodynia could result from either the prevention of central sensitization with the direct blockade of noxious stimuli, or from the blockade of low-threshold inputs [33]. Because zonisamide has Na^+ and T-type Ca^{2+} channel blocking properties, it was expected to significantly improve mechanical and chemical allodynia. However, Zonisamide consistently reduced mechanical allodynia caused by the touch of a smooth object (cotton bud) at the 80 mg/kg dose but was ineffective at the smaller doses. In case of chemical allodynia, both doses of zonisamide produce non-uniform trend of effects during whole treatment period (POD7 to POD13). The pathogenetic mechanisms responsible for these two symptoms (mechanical and chemical allodynia) associated with neuropathic pain are not the same, either in the rat CCI model or in human neuroapthic pain [34, 35]. It is possible that

mechanical injury (CCI) produces ischaemia of nerve fibres resulting in pain where as chemical allodynia produces pain due to chemokine release. The effect of ischaemia on ion channels is well known [9-11, 36, 37]. Hence, differences in the response of various symptoms of neuropathic pain to the same therapy have been seen [38]. Therefore, the different effects of zonisamide on these symptoms seen in the present study are not surprising and are consistent with the presumed different etiologies for these neurological phenomena. Unlike the effect of zonisamide on spontaneous pain, the effect on mechanical and chemical allodynia was not prolonged. Standard drug pregabalin showed dose dependent effectiveness. It was effective on lower dose but the effect was observed after three repeated administration (from POD9). The continuous post treatment antiallodynic effect was not found in case of both drugs.

Zonisamide reduced hyperalgesia (mechanical and thermal) in CCI rats. Zonisamide at 50 mg/kg, consistently reduced mechanically induced hyperalgesia and inconsistently did so at the higher dose during treatment study. Zonisamide seems to be effective in reducing thermal hyperalgesia in CCI rats. Zonisamide had a sustained effect at both doses without any carry-over effect after the treatment. Low-voltage-gated T-type Ca^{2+} channels are found in dorsal root ganglion primary afferent cell bodies and in free nerve endings. Here, these channels contribute to the initiation of the action potential in these locations by lowering the required threshold for activation. The fact that, T-type Ca^{2+} channel density has been increased in CCI rats [5-8], and by promoting burst activity and synaptic excitation, there is development of hyperalgesia [27]. Thus Na^{+} and T-type Ca^{2+} channel blocking property of zonisamide may contribute to reduce the hyperalgesia response. Standard drug pregabalin was also showed the same effect.

Recent findings proposed the role of microglial cells in neuropathic pain after the nerve injury. These cells are activated after peripheral nerve injury and regulate the synthesis and release of number of cytokines and chemokines including nitric oxide that contribute to the induction and maintenance of neuropathic pain by altering neuronal function [9-11]. Zonisamide inhibits the synthesis of nitric oxide [17], thus this may be the additional mechanism to reverse the neuropathic pain symptoms but further detail study is require to make any conclusion.

CONCLUSION

In conclusion, the present results indicate that zonisamide effectively exerts selective analgesic effects on neuropathic pain, as shown in the CCI rats. We propose that zonisamide should be considered as an alternative pharmacological tool for treatment of neuropathic pain due to a variety of causes that is largely refractory to standard analgesics such as pregabalin.

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