



## **Effect of mast cell stabilizer ketotifen on sciatic nerve ligation induced experimental neuropathic pain in Wistar rats**

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### **ABSTRACT**

The present study is designed to investigate the involvement of mast cells in neuropathic pain induced by partial sciatic nerve ligation. The effect was evaluated by assessing various behavioral parameters (thermal hyperalgesia, cold hyperalgesia), biochemical parameters (lipid peroxidation, reduced glutathione, superoxide dismutase and catalase). Partial Sciatic nerve ligation significantly caused thermal hyperalgesia, cold hyperalgesia and oxidative damage as compared to normal and sham group. Upon daily administration of ketotifen (5mg/kg, 10mg/kg) and gabapentin (100 mg/kg) considerably reversed hyperalgesia, cold hyperalgesia and also attenuated oxidative stress when compared to control group. The results indicated that ketotifen exerts ameliorative effect in neuropathic pain by inhibiting degranulation of mast cells and also by reducing oxidative stress.

**Keywords:** Neuropathic pain, mast cell, ketotifen, degranulation

### **INTRODUCTION**

Neuropathic pain is a complex and chronic pain state that is usually accompanied by nerve. The nerve fibers themselves may be damaged, dysfunctional, or injured. Neuropathic pain is different from nociceptive pain as it does not respond to conventional pain killers. Mast cells are considered significant in allergic disorders though they are also key initiators and effectors of innate immunity and are inhabitant in many tissues including the nerves [1, 2]. It has been widely known that mast cells get degranulated at the site of nerve lesion and release a cocktail of mediators [3,4] such as histamine, serotonin, proteases, prostaglandins and cytokines [1] but the exact mechanism of mast cell activation by nerve injury still remains unknown [5,6]. Numerous mediators of mast cells have the ability to sensitize nociceptors, including histamine [7,8]. Histamine also has well-known chemoattractant properties [9-11]. It is also capable of neutrophil recruitment, which on arrival further contribute to the release of algescic and inflammatory mediators. It has been reported that mainly H1 [12,13] and H4 [14,15] histamine receptors interfere with pain and inflammation. Therefore it can be deduced that H1 antagonists could possibly inhibit pain and inflammation [16,17]. Thus based on the above

cited indication, the objective of the present study was to assess the role of Ketotifen in neuropathic pain induced by partial sciatic nerve ligation (PSNL) in wistar rats [18,19].

### **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, I.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.* [20] 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. Behavioral estimations were done on 7<sup>th</sup> and 14<sup>th</sup> day. At the end of study (i.e. on 14<sup>th</sup> day) the rats were euthanized for collection of nerve tissue for biochemical estimations [21].

**Drug Treatment schedule:** The animals were divided into seven 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. Gabapentin 100 mg/kg, Ketotifen 5mg/kg, Ketotifen 10 mg/kg, Gabapentin 100 mg/kg+ Ketotifen 10 mg/kg were treated as group 4-7 respectively. In this experiment, the following groups of eight rats each were administered drugs once daily orally for the duration of 14 days. All the groups have undergone behavioral and biochemical tests. Doses of gabapentin and ketotifen were selected based on reported literature [22,23].

#### 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 7<sup>th</sup> day after induction of neuropathic pain and on the 14<sup>th</sup> 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 [24].

**b) Cold hyperalgesia** Cold hyperalgesia was assessed by measuring paw (both ipsilateral and collateral) withdrawal latency (PWL), when dipped in water bath which was maintained at 4°C ± 2°C on 7<sup>th</sup> and 14<sup>th</sup> day after PSNL[25]. A cut off time of 15 sec was maintained throughout the experimental protocol [26].

#### Biochemical estimations

**a) Dissection and homogenization:** Behavioral assessments were done on the 14<sup>th</sup> 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 were 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* [27]. 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 ( $1.56 \times 10^{-6} \text{m}^2/\text{cm}^2$ ) and expressed as n moles formed per mg of protein in the tissue [28].

#### 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 phosphomolybdate by tyrosine and tryptophan present in the protein [29].

**d) Estimation of reduced glutathione** Reduced glutathione concentration was anticipated according to the method illustrated by Ellman [30]. 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 ( $1.36 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ ) and expressed as percentage moles ( $\mu$  moles) per gram of tissue weight.

**e) Superoxide Dismutase (SOD)** was estimated according to the method defined by Marklund and Marklund [31]. The supernatant was assayed for SOD activity by subsequent the inhibition of pyrogallol autoxidation. 100 micro liters ( $\mu$ l) of cystolic supernatant was added to Tris HCL buffer. At least 25  $\mu$ l 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  $\mu$ l 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 [32].

#### Statistical Analysis:

All the results are expressed as mean  $\pm$  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  $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$  on 7<sup>th</sup> & 14<sup>th</sup> day after PSNL. A cut off time of 15 sec was maintained throughout the experimentation procedure. The PWL was evaluated on 7<sup>th</sup> day as well as on 14<sup>th</sup> day. Normal control group was found to be significant when compared to the PSNL group. Ketotifen (5 mg/kg and 10 mg/kg) significantly ( $P < 0.01$ ) increased the paw withdrawal latency time as compared to PSNL group on 7<sup>th</sup> and 14<sup>th</sup> day (Figure 1 & 2).

**Effect on Hot Plate:** Thermal hyperalgesia was measured by placing each animal on a hot plate (Eddy's Hot Plate) maintained at  $55^{\circ}\text{C}$  throughout the experimental procedure. Thermal hyperalgesia was assessed on weekly intervals on (7<sup>th</sup> & 14<sup>th</sup> 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 [33]. Normal control group was found significant when compared to PSNL group. While ketotifen (5 & 10 mg/kg), gabapentin (100 mg/kg) significantly ( $P < 0.01$ ) increased the jumping time when compared against PSNL group (Figure 3 & 4).

**Effects of Ketotifen on oxidative damage:** Sciatic nerve ligation significantly caused oxidative damage as indicated by increased lipid peroxidation (Figure 5), depletion of reduced glutathione level (Figure 6), superoxide dismutase (Figure 7) and catalase (Figure 8) activity in sciatic nerve. Treatment with ketotifen (5 & 10 mg/kg), gabapentin (100 mg/kg) and the combination of Ketotifen (10 mg/kg) and gabapentin (100 mg/kg) significantly ( $P < 0.01$ ) reverse the oxidative stress by decreasing the lipid peroxidation and increasing the reduced glutathione, SOD and catalase level.

## DISCUSSION

Neuropathic pain is the most intense of all the pains which could arise due to nerve lesion [21]. The management of neuropathic pain has been

unsuccessful because of its resistance to conventional pain killers such as non-steroidal anti-inflammatory drugs [22]. Thus there arises 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 in neuropathic pain and to understand the involvement of mast cells in pathophysiology neuropathic pain. In this study partial sciatic nerve ligation (PSNL) model was used to induce neuropathic pain in wistar rats.

Post surgical behavioral signs such as spontaneous pain in the form of paw guarding and licking on the injury site have been reported. The behavioral alterations like cold hyperalgesia, chemical hyper-reactivity, and mechanical hyperalgesia have also been reported to take place within a week after surgery [9,10, 23]. 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 tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [34] resulting in increased firing rates & activation of nociceptors. Ketotifen stabilizes mast cell by reducing their degranulation and decreasing release of various mediators as well as reactive oxygen species generation by phagocytes involved in pathophysiology of neuropathic pain by reducing transmembrane influx of calcium ions.

It also has non-specific anti-inflammatory action and also has ability to neutralize reactive oxygen species. The results of the present study clearly showed that treatment with ketotifen (5 & 10 mg/kg) significantly attenuated sciatic nerve ligation induced behavioral alterations in pain perception. Further treatment with gabapentin (100 mg/kg) along with ketotifen (10 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.

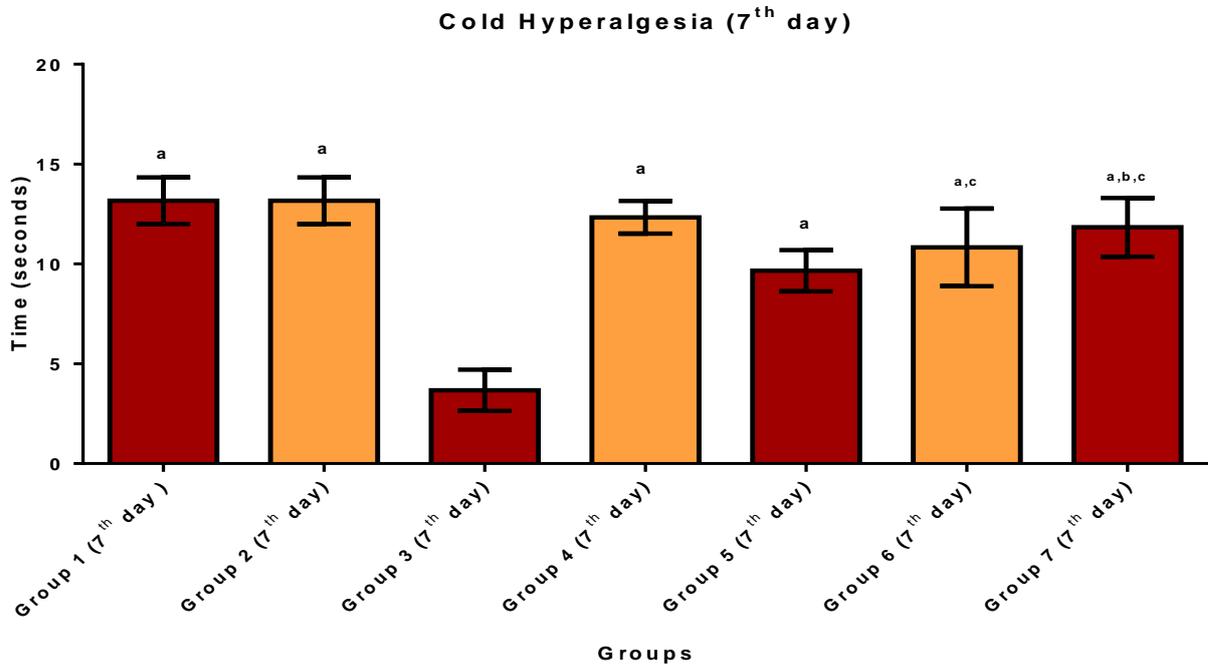
Ketotifen (5 & 10 mg/kg) alone and ketotifen (10 mg/kg) in combination with gabapentin (100

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 [35]. The results of the present study evidently demonstrate the involvement of mast cell degranulation and antioxidant mechanism in the protective action of ketotifen against the neuropathic pain induced by partial sciatic nerve ligation [23].

**Conclusion**

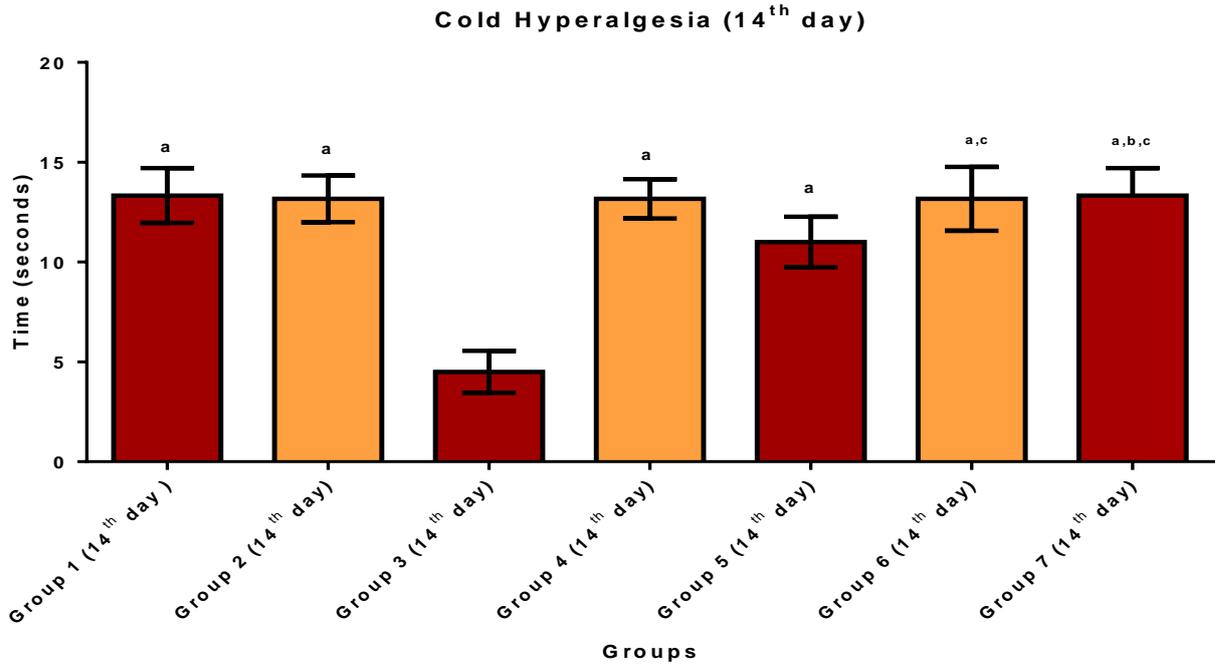
In conclusion, the present study suggests that ketotifen stabilizes mast cells and reduces the release of various inflammatory mediators which is responsible for the alleviation of symptoms of neuropathic pain. Ketotifen (5 & 10 mg/kg) alone and ketotifen (10 mg/kg) in combination with gabapentin (100 mg/kg) significantly reduced the levels of lipid peroxidation and restored the low levels of glutathione, catalase and superoxide dismutase and also increased the paw withdrawal latency time in hot plate and cold hyperalgesia examinations. Thus, the current study demonstrated the involvement of mast cells in neuropathic pain.

**Figure 1- Effect of Ketotifen and gabapentin on Cold Hyperalgesia ( 7<sup>th</sup> day)**



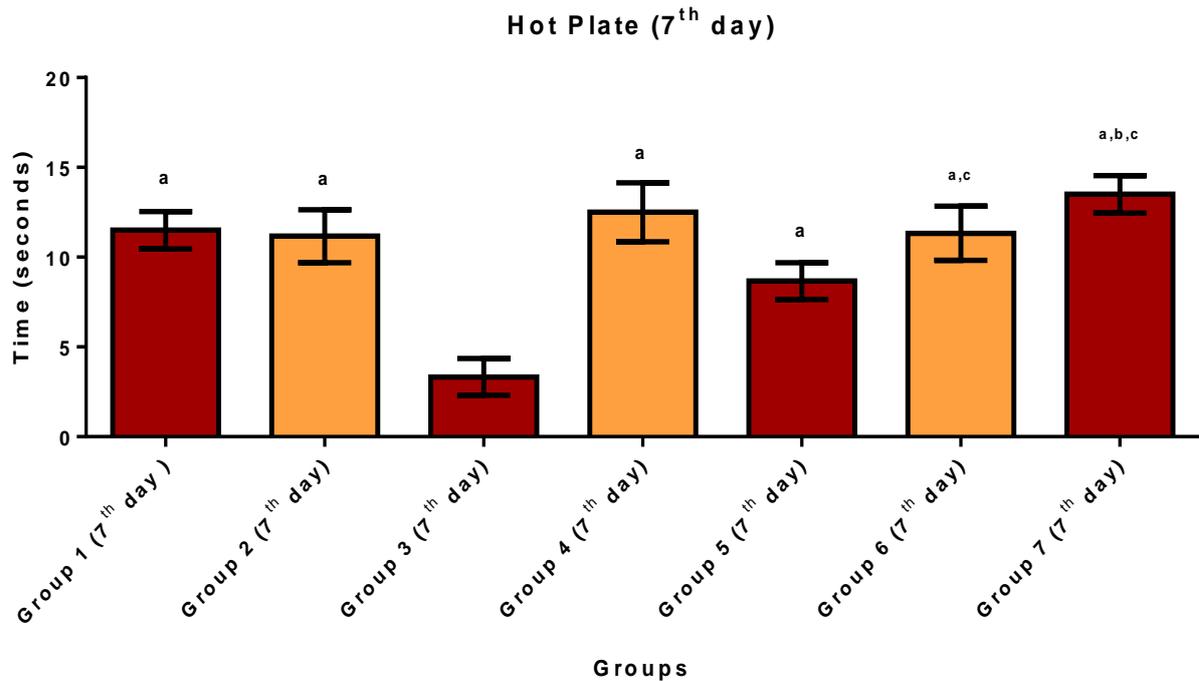
All values were expressed as Mean ± S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett’s test).

Figure 2- Effect of Ketotifen and gabapentin on Cold Hyperalgesia ( 14<sup>th</sup> day)



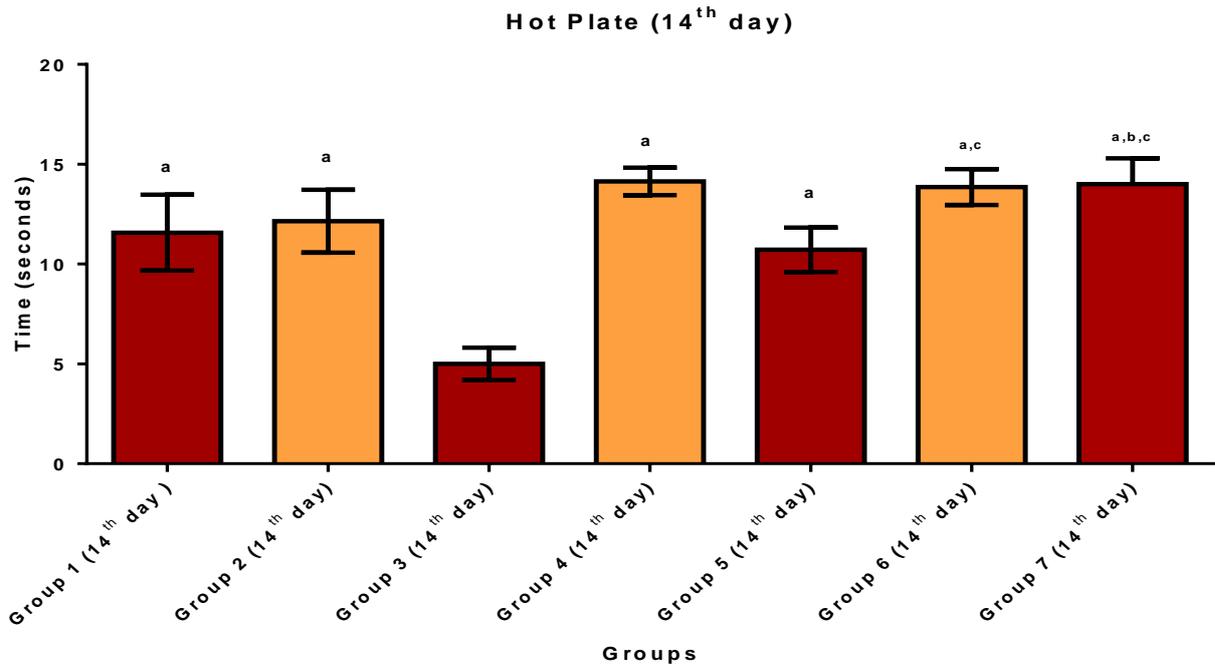
All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 3 - Effect of Ketotifen and gabapentin on Hot Plate ( 7<sup>th</sup> day)



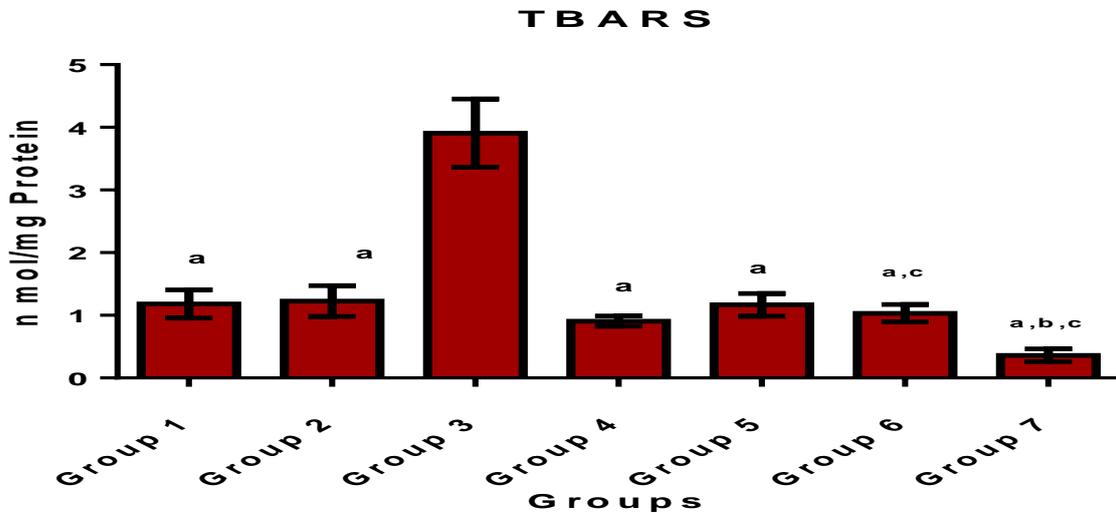
All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 4- Effect of Ketotifen and gabapentin on Hot Plate ( 14<sup>th</sup> day)



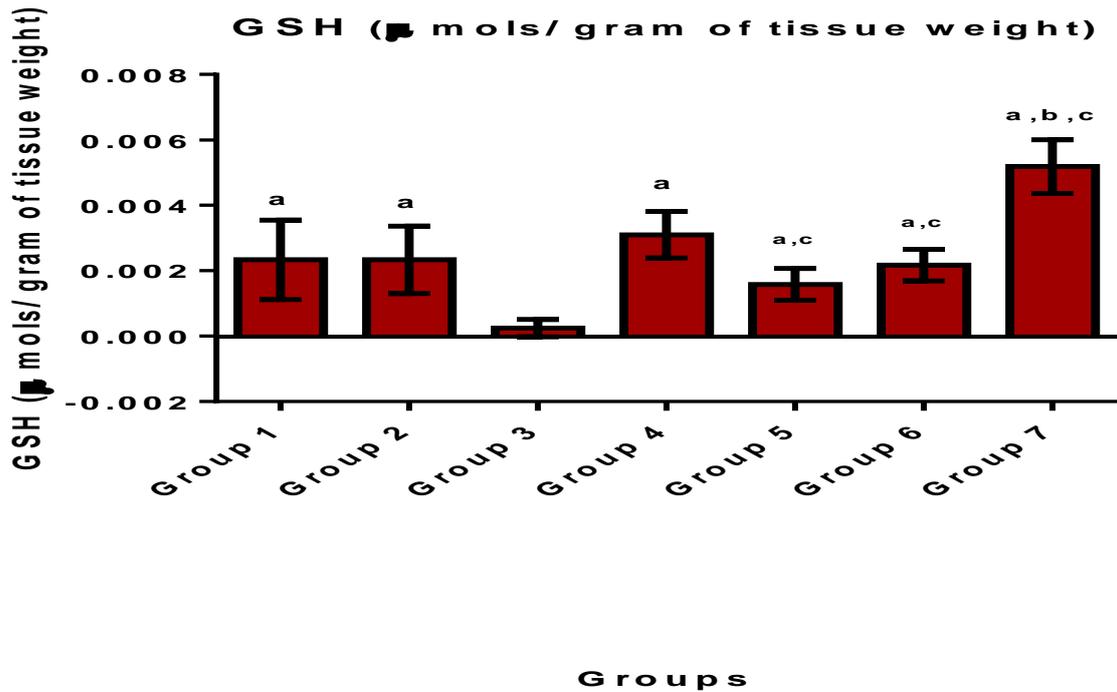
All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 5- Effect of Ketotifen and gabapentin on Lipid Peroxidation in sciatic nerve



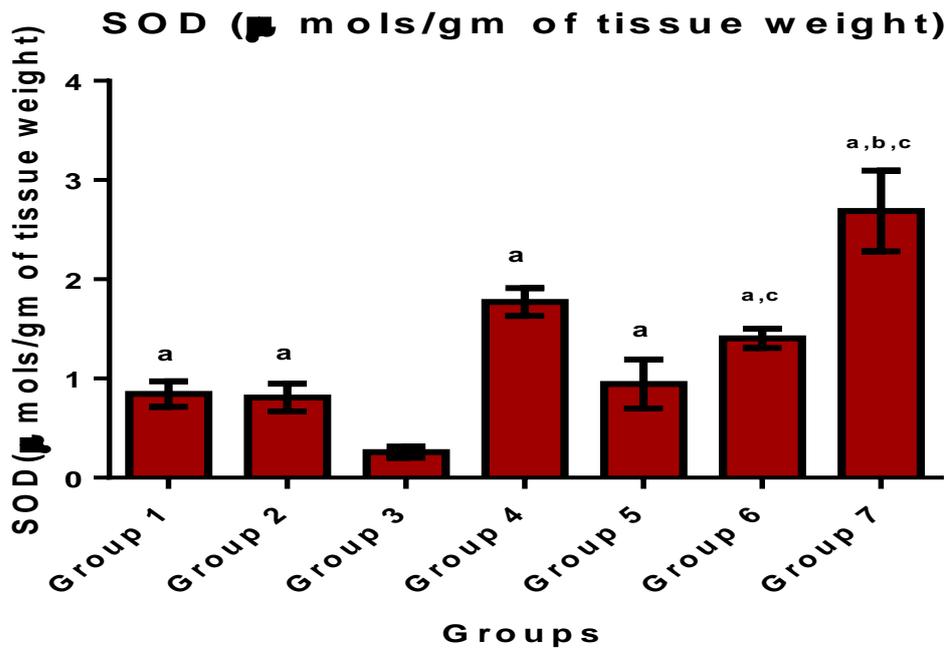
All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 6- Effect of Ketotifen and gabapentin on Reduced Glutathione in sciatic nerve



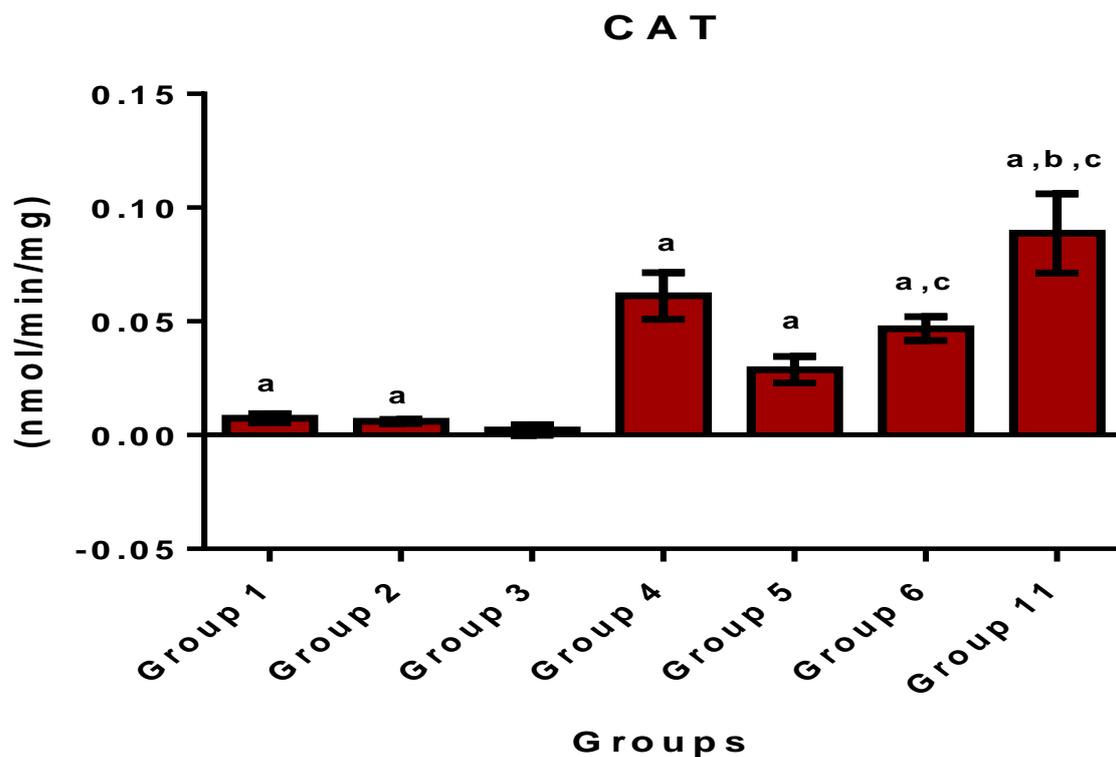
All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 7- Effect of Ketotifen and gabapentin on Superoxide Dismutase in sciatic nerve



All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

Figure 8- Effect of Ketotifen and gabapentin on Catalase in sciatic nerve



All values were expressed as Mean  $\pm$  S.E.M. (n=8); <sup>a</sup>= P<0.01 when compared with PSNL; <sup>b</sup>= P<0.01 when compared to Ketotifen 10 mg/kg.; <sup>c</sup>= P<0.01 when compared to Gabapentin 100mg/kg (ANOVA followed by Dunnett's test).

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