Journal of Neurological Sciences [Turkish] **29:(1)**# 30; 065-075, 2012
http://www.jns.dergisi.org/text.php3?id=498
Research Article

Ketorolac Tromethamine Promotes Functional Recovery and Enhances Nerve Regeneration After Sciatic Nerve Trauma in Rats

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Summary

Objective: The aim of this study is to investigate the effects of ketorolac tromethamine (KT) in sciatic nerve trauma model.
Material and Methods: Twenty-seven Wistar rats with normal motor activity were used. Group Sham (n:3): Surgical procedure without sciatic nerve trauma was applied. Group S (n:12): One mL saline was administered intraperitoneally four times at 6 hours intervals after nerve trauma with an aneurysm clip. Group KT (n:12): One mL 50 mg/kg KT was administered intraperitoneally in the same time periods. 24 hours after nerve trauma, subjects were randomised into 2 groups as acute and chronic. The footprints of rats were scanned in acute group for 3 days and in chronic group on days 1, 2, 3, 7, 14, 21, 28, 35 and 42. Static sciatic index (SSI) was calculated. Sciatic nerve sections were assessed histopathologically.
Results: When SSI mean values were compared there were no significant difference between chronic groups until 42. day. But on 42. day in Group KT SSI mean value was significantly higher ($p<0.05$). In acute Group KT; axon diameter mean values were significantly higher and fibroblast count mean values were significantly lower than Group S ($p<0.05$). In chronic Group KT; axon diameter, myelin diameter, axon count mean values were significantly higher ($p<0.05$) and fibroblast and mast cell count mean values were significantly lower than Group S ($p<0.05$).
Conclusion: In sciatic nerve trauma model, we have established that four times intraperitoneal 50 mg/kg of KT is associated with improvement of motor function.

Key words: Peripheric nerve trauma, ketorolac tromethamine, static sciatic index, mast cell, fibroblast

Ketorolak Trometamin Ratlarda Siyatik Sinir Hasarı Sonrası Fonksiyonel İyileşme ve Sinir Rejenerasyonunu Arttırır

Özet

Giriş: Bu çalışmada siyatik sinir travma modeli oluşturulan ratlarda ketorolak trimetaminin (KT) etkinliğini araştırmak amaçlandı.

Materyal-Metod: Çalışmaya 27 adet normal motor aktiviteye sahip Wistar albino rat alındı. Grup Sham (n:3): Sinir travması oluşturulmadan sadece cerrahi işlem yapıldı. Grup S (n:12): Anevrizma klempi ile oluşturulan sinir travması sonrası 6 saatte bir 4 kez 1 mL salin intraperitoneal olarak verildi. Grup KT (n:12): Sinir travması sonrası aynı zaman aralıklarında intraperitoneal 50 mg/kg KT verildi. Sinir travmasından 24 saat sonra denekler akut ve kronik olmak üzere iki gruba ayrıldı. Ratların ayak izleri akut grupta 3 gün, kronik grupta ise 1, 2, 3, 7, 14, 21, 28, 35 ve 42. günlerde tarayıcı ile tarandı. Statik siyatik indeksi (SSI) hesaplandı. Siyatik sinirlerden alınan kesitler histopatolojik olarak incelendi.
Bulgular: Kronik grupta gruplar arasında SSI değerleri ortalamaları karşılaştırıldığında; 42. güne kadar anlamlı fark bulunmazken, kronik Grup KT' de 42. gün SSI ortalama değerinde anlamlı artma saptandı ($p<0.05$). Akut Grup KT' de akson çapı ortalama değerleri anlamlı yüksek ve fibroblast sayısı ortalama değerlerinde anlamlı azalma bulundu ($p<0.05$). Kronik Grup KT' de akson çapı, miyelin çapı, akson sayısı ortalama değerleri anlamlı yüksek bulundu ($p<0.05$). Kronik Grup KT' de fibroblast sayısı ve mast hücre sayısı ortalama değerlerinde anlamlı azalma bulundu ($p<0.05$).
Sonuç: Siyatik sinir travma modelinde intraperitoneal yolla 4 kez uygulanan 50 mg/kg KT'in erken dönemde; sadece akson çapında artma ve fibroblast sayısında azalma sağlarken, geç dönemde; akson çapının yanısıra miyelin çapı ile akson sayısında artış, motor fonksiyonda iyileşme, travma bölgesinde fibroblast sayısının yanında mast hücre sayısında azalma oluşturduğunu saptadık.

Anahtar Kelimeler: Periferik sinir travması, ketorolak trometamin, statik siyatik indeksi, mast hücresi, fibroblast

INTRODUCTION

Today, there are new developments on surgical treatment of peripheral nerve injury and multi-disciplinary therapeutic approach render this topic more attractive. Particularly traumatic peripheral nerve injury involves physical, psychological and socioeconomical problems[23]. There are in excess of 50.000 peripheral nerve repair procedures performed each year in USA[8]. We could not detect any statistics on this so far.

When nerve is injured, there might be functional loss of mild paresthesia to complete loss or mild muscle weakness to total paralysis. Severity of the neural lesion is determined by the amount of pressure, duration and applied area[24].

Understanding of neural structure, regeneration and factors inhibiting regeneration might increase the success rate of surgical repair. Today there are new developments in treatment of peripheral nerve injury[22]. New advances in molecular biology might provide better options in reconstructive surgical repair of peripheral nerve. Non-steroid anti-inflammatory agents, steroids, nerve growth factors, erythropoietin, thyroid hormones, growth hormone, adrenocorticotrophic hormone, insulin-like peptides are used for experimental peripheral nerve injury[7,14,23,26].

Ketorolac tromethamine (KT), a pyrolacetic acid derivative, is a nonsteroidal antiinflammatory drug (NSAID) and is frequently preferred for the management of postoperative pain, renal colic, sickle cell crysis, arthritis, lumbalgia, headache and cancer pain either intrathecally or intramuscularly[14]. Ketorolac tromethamine has also been reported to provide successful analgesia when used via intracerebroventricular, intrathecal, and epidural routes in experimental models[16,18]. It's optimal doses can be used at the middle or severe pain alternatively to the opioids[21].

Peripheric tissue damage or inflammation causes the release of inflammatory mediators like prostaglandins (PG).

Ketorolac tromethamine inhibits the PG synthesis, so that peripheric nerve tissue stimulation and feel of pain decreases. Nonsteroidal antiinflammatory drugs inhibit Cyclo-oxygenase (COX) 1 and 2[21]. Cyclo-oxygenase inhibitors prevent especially PGE_2 and $PGF_{\alpha 2}$ induced hyperalgesia. COX inhibitors can control the body temperature and acute/chronic inflammation[18]. Padi et al[18] showed that 30 mg kg^{-1} intraperitoneal (IP) KT can suppress the nociseptive behavior. It has been shown that in rat spinal cord ischemia and trauma model intrathecal (IT) KT usage decreases the dimension of the lesion and spinal nerve damage, improves the motor function[11].

The aim of this study was to investigate the effects of ketorolac tromethamine on sciatic nerve static index, axon and myelin diameter, quantity of fibroblasts and mast cells in the sciatic nerve trauma model in rats.

MATERIAL AND METHODS

The study protocol was approved by the Animal Research Committee of the University of Dokuz Eylul. Twenty- seven Wistar rats with normal motor activity, weighing between 200-250 g, were used in this study. Test animals in group K received Ketrodol (50mg/kg/24 h, 4 times at 6 hours intervals) (n:12), group S received normal saline (for 4 times at 6 hour intervals) (n:12), and group sham animals (no surgery) (n:3)[16,18,21].

Surgical procedures

After induction of anaesthesia with 40 mg/kg of IP thiopenthal, rats were positioned on the right lateral side, their left thigh was shaved and the skin was prepared with %10 povidon-iodine. The left sciatic nerve was exposed by fascial plane via a longitudinal incision. The sciatic nerve was exposed at mid-thigh level (Figure 1) and crushed for two minutes with the aneurysm clip (Aesculap, Yaşargil standard aneurysm clip, FE783, Tuttlingen, Germany) which exerts 63 gram force [0.6 Newton (N)][7] (Figure 2). Then the clip was removed and the wound was sutured in layers and the rats were allowed to recover. All the operations were performed under the microscope by same surgeon.

KT was supplied by DEVA Laboratories in sterile packages without preservatives (MW 376.41 Dalton, Ketrodol, DEVA, İstanbul, Turkey). KT was diluted with % 0.9 NaCl with a pH between 6.5-7. One mL of 50 mg/kg ketorolac tromethamine was administered intraperitoneally 4 times at 6 hours intervals after trauma has been performed.

Static Sciatic Index measurement:

After drug administrations, animals were divided randomly into 2 groups as acute or chronic. 24 hours after traumatic sciatic injury, in order to measure static sciatic index (SSI), the rats were placed on a digital scanner (HP PSC 1215, Hewlet-Packard Inc, Malaysia)[4] (Figure 3) and then covered with a white box with several holes on the top for air circulation.

The scanning process was started 5 minutes after the animal was placed on the scanner surface in order to adapt the environment. For better scanning quality, all lights were closed during the scanner. The examination was repeated when the animal moved (Figure 4).

In the acute group, we scanned footprints of rats every day at the same hour for 3 days. In the chronic group, we scanned footprints of rats every day at the same hour for 3 days, then once a week for 42 days (1, 2, 3, 7, 14, 21, 28, 35, 42. days)[2].

From the footprint images of rats, toe spread (TS) and intermediate toe spread (ITS) were measured.

Toe spread (TS) : Distance between first and fifth toes

Intermediate toe spread (ITS) : Distance between second and fourth toes

Measurements are named as; experimental toe spread (ETS), experimental intermediatetoe spread (EITS) for operated foot, and as non-operated toe spread (NTS), non-operated intermediatetoe spread (NITS) for non-operated foot. Then toe spread function (TSF) and intermediate toe spread function (ITSF) were calculated and used to calculate SSI. A total of 0 indicates normal nerve function and progressive loss of function is indicated by a negative score[2,3].

TSF : (ETS-NTS)/NTS

ITSF: (EITS-NITS)/NITS

SSI = 108,44 TSF + 31,85 ITSF - 5,49

Histomorphometry:

At the end of the study (acute group at the end of 3 days, chronic group at the end of 42 days) the rats were sacrificed under high dose thiopental, followed by intracardiac perfusion with 10% buffered formaldehyde, and traumatized sciatic nerve was excised. The sciatic nerve was examined with routine histological tissue follow-up. Traumatized parts of sciatic nerve were embedded into paraffin blocks and cut slices of 1 µm thickness with Reichert Rotary microtome (Leica, Köln, Germany). After the staining process with toluidin blue, the preparations were assigned randomly. The preparations were examined with light microscope (Olympus BH-2, Tokyo, Japan) x 100. We recorded with video camera (JVC Colour Video Camera Head Model No: TK-890E, Tokyo, Japan) and transferred these video images into computerized digital environment using a program (AverMedia, AverTV WDM Video Capture, Milpitas,California, USA). We shot the digital photos of suitable preparations and measured diameters of axon and myelin using the image analysis program University of Texas Health Science Center at San Antonio Image Tool for Windows Version 3.00 (developed at the University of Texas Health Science Center at San Antonio, TX, USA). Besides, every axon, fibroblast and mast cells were counted in 2500 μm^2 area.

Statistical Analysis:

We used statistical analysis with 11.0 version of SPSS for Windows. Results are written as mean±standard deviation. Intergroup analysis was performed with Kruskal-Wallis and Mann-Whitney U test. $p< 0.05$ is accepted as statistically significant.

Figure 1: *Left sciatic nerve on the right lateral side*

Figure 2: *Aneurysm clip on the left sciatic nerve*

Figure 3: *Rat on the scanner*

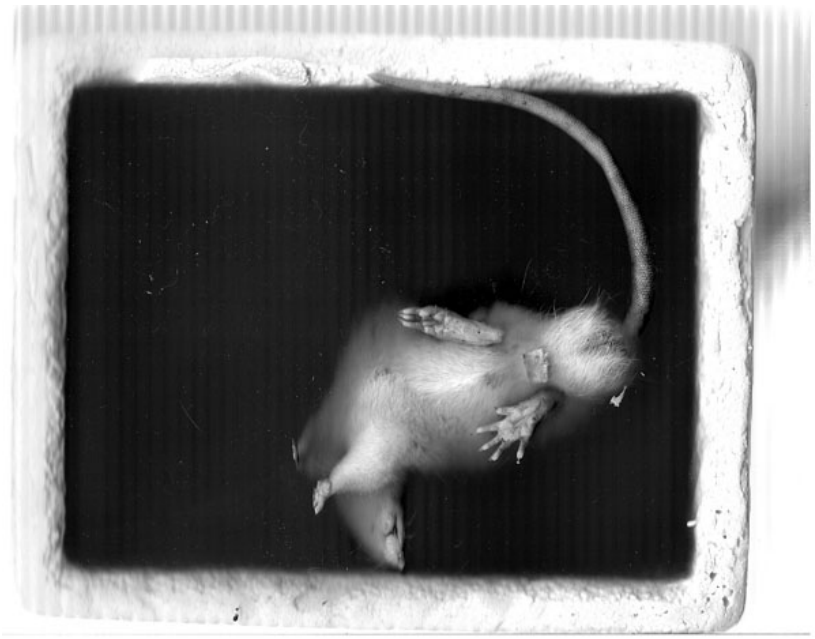

Figure 4: *The scanner image of the rat with sciatic nerve trau*

RESULTS

Preoperatively, the SSI in rats in both groups was approximately zero, indicating normal function. After injury, the SSI decreased to approximately -100, indicating complete loss of function. The SSI values as an indicator of functional recovery are listed in Table 1 and 3.

In acute groups, comparison of average SSI values; 1, 2 and 3th days revealed no statistical significance (Table 1). Significant statistical difference was found between the 2 acute groups with respect to the axonal diameter (p=0.004) and fibroblast number average values (p=0.002). Nerves treated with KT had higher axonal diameter and fewer fibroblast number average values (Table 2). In contrast, there were no statistical significance according to myelin diameter (p=0.699), axonal counts (p=0.589) and mast cell count average values (p=0.937) (Table 2).

In the initial recordings at 5 weeks after the operation; the average SSI values were similar in the 2 chronic groups. At the sixth week, the values were significantly better in group K than group S (p=0.009) (Table 3).

Histomorphometry

Chronic groups K and S were compared according to axonal diameter, myelin diameter, axonal number average values. Group K was significantly higher in all parameters (Table 4).

In addition, chronic groups K and S were compared according to fibroblast number and mast cell count average values. Chronic Group K fibroblast number and mast cell count average values were significantly lower (Table 4).

Light microscope revealed acute and chronic S groups, also acute Group K were associated with diffuse axonal regression, myelin degeneration, but there were decreased axonal regression and myelin degeneration in chronic Group K.

Table 1: Acute Group average SSI values

SSI	Acute Group SF (n: 6)	Acute Group KT (n: 6)	p
1st day	-95,83±4,03	-90,99±5,51	0.132
2nd day	-89,08±7,15	-86,12±6,20	0.310
3rd day	-87,44±4,01	-80,25±9,27	0.093

Table 2: Acute groups histomorphometric average values

	Sham Group (n:3)	Acute Group SF (n:6)	Acute Group KT (n:6)	p
Axon diameter (µm)	6.57±0.50	4.58±0.37	5.34±0.43*	0.004
Miyelin diameter (µm)	2.15±0.11	1.69±0.18	1.76±0.19	0.699
Axon number	26.42±0.89	15.30±4.30	17.03±3.94	0.589
Fibroblast number	1.31±0.24	3.37±0.68	1.97±0.41*	0.002
Mast cell number	0.08±0.12	0.43±0.30	0.34±0.15	0.937

* p<0.05 Inter-Group Comparison

Table 3: Chronic Group average SSI values

SSI	Chronic Group SF (n: 6)	Chronic Group KT (n: 6)	p
1. day	-91,19±8,89	-82.31±12.14	0.240
2. day	-75,96±23,87	-75.96±21.91	0.937
3. day	-75,03±12,19	-67,86±12,68	0.310
7. day	-74,10±21,49	-56.55±22.12	0.310
14. day	-35,54±22,16	-32,04±18,00	0.699
21. day	-17,76±9,65	-10.29±6.36	0.180
28. day	-12,30±4,92	-9,39±7,88	0.485
35. day	-7,32±5.23	-3.65±2.41	0.310
42. day	-6,97±4.72	-2.02±1.66*	0.009

* p<0.05 Inter-Group comparison

Table 4: Chronic group histomorphometric average values

	Sham Group (n:3)	Chronic Group SF (n:6)	Chronic Group KT (n:6)	P
Axon diameter(µm)	6.57±0.50	4.48±0.69	6.31±0.46*	0.004
Miyelin diameter (µm)	2.29±0.11	1.73±0.16	2.15±0.18*	0.004
Axon number	26.42±0.89	14.92±3.16	21.94±2.28*	0.009
Fibroblast number	1.31±0.24	5.15±1.33	1.77±0.93*	0.002
Mast cell count	0.08±0.12	0.31±0.17	0.13±0.10*	0.026

* p<0.05 Inter-Group comparison

DISCUSSION

In our study; sciatic nerve trauma model revealed that in early phase IP administered KT provides increased axonal diameter, whereas in late phase increased axonal and myelin diameter, also increased axon number with improved motor functions according to static sciatic index. Besides, in both early and late phases fibroblast numbers decreased and mast cell count decreased in late phase.

In our study; we used aneurysm clip with closing pressure 63 gram (0.6 N) for 2 minutes. We induced sciatic nerve damage and showed trauma of Sunderland type II injury. Likewise, in the literature it has been showed that functional sciatic nerve injury was induced by two minutes of 53 gram aneurysm clip[1,7].

In our study; we chose the maximum effective analgesic dose of 50 mg kg^{-1} ketorolac from the spectrum of 4.5-120 mg kg^{-1}. Since its half life is about 6 hours, we administered 50 mg kg^{-1} IP ketorolac every 6 hours in 24 hours[16,18,21].

In this study; we induced peripheral nerve trauma in sciatic nerve and performed functional evaluation at 1st, 2nd, 3rd, 7th, 14th, 21st, 28th, 35th and 42nd days with scanner. For functional evaluation, Bervar et al.[3] recorded rat footprints at 1st, 2nd, 3rd, 7th, 14th, 21st, 28th, 35th and 42nd days with video camera and calculated SSI. They suggested that SSI calculation is a convenient method of motor function evaluation after peripheral nerve injury. In the light of these literature information, we chose reliable SSI method to monitor sciatic nerve functional recovery and it also shows positive correlation with histologic and histomorphometric findings[3,9].

In Sunderland type II nerve injury; there is axonal damage, endoneural sheath is intact, there is distal Wallerian degeneration[15,27]. After this type of neural damage spontaneous regeneration begins and functional recovery will be sufficient. We performed axonotmesis or Sunderland type II damage and showed that in group K rats begin to walk normally at 30-35 days but in group S 35-42 days. In accordance, Lu'is et al[15] reported that SSI returns preoperative values in 7 weeks on the peripheral nerve model which is traumatized with a clamp of 54 N closing pressure for 30 seconds.

After the nerve is traumatized, there are changes on both sides of the lesion. There is edema at the distal part of the axon and beginning from the lesion destruction proceeds distally. Then, chromatolysis occurs together with histological changes

like edema at stem[13]. Although regeneration is effective at peripheral nervous system, full functional recovery is not often. During functional recovery of nerve, regenerating axon should reach distal target. Axonal regeneration is 2-3.5 mm day^{-1}[8]. Galtrey et al[8] reported beginning of recovery at 14-56 days; functional recovery at 21-42 days. Before full functional recovery, first axonal diameter and thickness of myelin sheath should return normal values. Although there is axonal damage after trauma; if Schwann cell, basal lamina and perineurium are intact regeneration will be easier[13]. In our study; we showed that IP route ketorolac leads to functional recovery at 42nd day. Although there were no significant difference in early acute phases, SSI values in Group K were closer to normal. Likewise, in late chronic phases except 42nd day SSI values were not significantly different, but SSI values of Group K were closer to normal. We attribute this result to anti-inflammatory effect of KT which inhibits non-selectively COX-1 and COX-2 enzymes, thereby preventing PG synthesis, decreasing microvascular permeability and edema.

COX-2 enzyme increase after trauma enhances PG synthesis and aggravates damage at the traumatized tissue. Likely, Durrenberger et al[5,6] showed that COX-2 enzyme increase after CCI chronic constriction injury at the Sunderland type II region leads to Wallerian degenerasyon. COX-2 inhibition might effect regeneration. Parris et al[19] showed in a rat CCI model that IT route KT prevents PG biosynthesis mechanism, thereby having anti-nociceptive effect.

Gupta et al[10] showed that after CCI at the trauma region, apoptosis and Schwann cells build up, large diameter axons decrease and small diameter axons increase. Traumatized peripheral nerve remyelinisation index might be smaller myelin diameter and changes in myelin structure. Changes in axon diameter triggers Schwann cell build up and alters myelin thickness[10]. Abnormally smaller myelin thickness according to axon diameter is accepted as index of remyelinisation[12]. In our study, ketorolac increased axon diameter in acute phases, but in late chronic phases axon and myelin diameter increased, also axon number increased. Furthermore, we found out that axon and myelin diameter, also axon number did not reach normal nerve average values at 42nd day. Similarly Varejao et al[27] showed that regenerating myelin sheaths are smaller and thinner. Even at the post-operative 56th day regenerating nerves' density, number, size, myelin thickness could not reach normal nerve values. Lu'is et al[15] showed that 84 days after peripheral nerve trauma, its size was still smaller than normal, fiber density and number was increased. End-to-end peripheral nerve repair models show that fiber number increases in first 3 months then decreases gradually. The reason of delayed decrease in nerve density and number is dying of collateral fibers which could not reach distal appropriate targets[15].

Mast cells are also main cells of immunity as well as allergic reactions. Mast cells at the distal part of the peripheral nerve trauma are activated, they secrete proinflammatory mediators like histamine, serotonin, cytokines, proteases, PG, neurotrophins. These mediators cause neuropathic pain after trauma[25]. In our study; according to sham group, mast cells increased in both acute and chronic phases.

We showed that ketorolac decreased mast cell number to normal values in late phases. Ketorolac trometamin has powerful effect at nociceptive nerve endings and axons. We suggest that ketorolac effects mast cell membrane, decreases degranulation and neurogenic inflammation, also it suppresses secondary mast cell activation. It has been reported that tissue edema and mast cell density can be prevented by cromoglycate which is the

mast cell membrane stabilizator. If mast cells are stabilized, migration of neutrophils and macrophages can be stopped[28].

After peripheral nerve trauma, collagen and other connective tissue components increase particularly at epineurium[17,28]. Connective Tissue Growth Factor mediates cellular functions like adhesion, fibroblast build up and matrix formation. We showed that ketorolac decreased fibroblast number at the injury site in both acute and chronic phases. Ketorolac trometamin suppresses mast cell activation, decreases migration of fibroblasts, as a result inhibits fibrosis. Therefore we suggest that functional motor recovery might be better. Similarly, Gupta et al[10] reported that after CCI axonal diameter of large axons decrease because of large connective tissue build up. This connective tissue mass might pressurize axons at the region.

CONCLUSION

In our sciatic nerve trauma model study; intraperitoneal 50 mg kg^{-1} ketorolac trometamin 4 times in 24 hours in acute phase provides axonal diameter increase and fibroblast number decrease at trauma region. In late phase; in addition to axonal diameter, myelin diameter and axon number were also increased. Motor functions improved, in addition to decreased fibroblast number at trauma region, mast cell numbers also decreased.

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Received by: 12 February 2012
Revised by: 29 February 2012
Accepted: 01 March 2012

The Online Journal of Neurological Sciences (Turkish) 1984-2012
This e-journal is run by Ege University Faculty of Medicine,
Dept. of Neurological Surgery, Bornova, Izmir-35100TR
as part of the Ege Neurological Surgery World Wide Web service.
Comments and feedback:
E-mail: editor@jns.dergisi.org
URL: http://www.jns.dergisi.org
Journal of Neurological Sciences (Turkish)
Abbr: J. Neurol. Sci.[Turk]
ISSNe 1302-1664

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