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RESEARCH ARTICLE

The Neurotoxic Effect of Intrathecal Diclofenac Sodium in Rats

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Summary

We aimed to investigate the possible neurotoxic effects of single and repeated doses of diclofenac sodium administered to rats. The current study included 24 male Sprague-Dawley rats weighing 250-300 g. At the end of a 4-h fasting period, the rats were randomly split into 3 groups following the establishment of anesthesia with intraperitoneal delivery of 100 mg kg^{-1} of ketamine hydrochloride and 10 mg kg^{-1} of xylazine chloride. Group 2 (n=8) received 10 µl (200 µg) of intrathecal diclofenac sodium on 7th day as a single dose, whereas the rats in group 1 (n=8) and group 3 (n=8) were received 10 µl of intrathecal 0.9% saline and 10 µl (200 µg) of intrathecal diclofenac sodium via a 0.40 x 50 mm needle per day for 7 days. During the course of the study the animals were examined with regard to clinical toxicity. After fixating the tissues by injection of 10% formaldehyde, spinal cords were explored and evaluated histopathologically with electron microscopy. Statistical analysis was performed with the Kruskal Wallis test. P values <0.05 were considered statistically significant. While electron microscopic examination showed no changes in the control group, diclofenac sodium exhibited neurotoxic effects that were more marked following the 7-days treatment. Diclofenac sodium was neurodegenerative, depending on the dose, and cellular organelles were observed to have compression associated with extracellular edema. Neurodegeneration was thought to be occured with a significant reduction in cellular activity.

Keywords: *Diclofenac sodium, Neurotoxic effects, Intrathecal*

Ratlarda İntratekal Diklofenak Sodyumun Nörotoksik Etkisi

Özet

Amacımız intratekal diklofenak sodyumun tek ve tekrarlayan uygulanmasının olası nörotoksik etkisinin araştırılmasıdır. Çalışmamız 24 adet 250-300 gr ağırlığında erkek Spraque Dawley cinsi ratta gerçekleştirilmiştir. Dört saatlik açlık süresi sonrasında, 100 mg kg^{-1} ketamin hidroklorür ve 10 mg kg^{-1} ksilazin hidroklorür intraperitoneal uygulanarak anestezi sağlanan ratlar randomize olarak üç gruba ayrıldı. Grup 2 (n=8)'deki ratlar 7. günde intratekal 10 µl (200 µg) diklofenak sodyum, Grup 1 (n=8)'e intratekal 10 µl %0.9'luk serum fizyolojik ve Grup 3 (n=8)'e intratekal 10 µl (200 µg) diklofenak sodyum her gün intratekal girişim 7 olacak şekilde lomber bölgeden 0.40x50 mm'lik iğne ile intratekal uygulama yapıldı. Çalışma süresince klinik nörotoksisite açısından hayvanlar incelendi. %10'luk formaldehid enjekte edilerek dokuların fiksasyonu sağlandıktan sonra medulla spinalisleri eksplore edildi ve histopatolojik olarak elektron mikroskobu ile değerlendirildi. İstatistiksel değerlendirmede Kruskal Wallis testi kullanıldı. P<0.05 anlamlı olarak değerlendirildi. Elektron mikroskobik incelemede; kontrol grubunda bir değişiklik olmazken, 7 gün tekrarlayan uygulamada daha bariz olmak üzere diklofenak sodyumun nörotoksik etkileri olduğu bulundu. Diklofenak sodyum uygulamasının doza bağlı olarak nörodejenerasyona neden olduğu, ekstrasellüler ödem ve buna bağlı olarak hücre organellerinde baskılanma oluşturduğu saptanmıştır. Nörodejenerasyonun hücre aktivitesinde belirgin azalmaya bağlı gelişebileceği kanısına varılmıştır.

Anahtar sözcükler: *Diclofenac Sodyum, Nörotoksik etki, İntraspinal*

INTRODUCTION

Pain has become one of the most important issues associated anesthesia, as it causes emotional disorders and negatively affects the circulatory, pulmonary, endocrine, gastrointestinal, and genitourinary systems [1]. In light of the fact that each year 4-5 million people die from cancer worldwide, pain and discomfort experienced by those patients in their last days stand out as a medical and social issue [2].

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Local anesthetics and opioids are the most commonly employed agents for intrathecal and epidural pain management, and more effective analgesia is achieved at lower doses. Use of low doses reduces the frequency of side effects associated with the administered agents; however, when opioids are used some side effects, such as respiratory depression, urinary retention, pruritus, tolerance, and sedation, can be observed [3]. These unwanted effects have led to the development of novel drugs to improve pain management.

Diclofenac is a commonly used intraoperative NSAID known to have analgesic action both in the peripheral tissues and in the CNS. It has been shown that spinal cord cyclooxygenase (COX)-1 plays an important role in the first nociceptive component of acute, postoperative pain, and that inducible spinal cord COX-2 is involved in the later inflammatory primary hyperalgesia [4]. Intrathecal diclofenac has a 100-fold antinociceptive potency when compared with systemic diclofenac [5].

Lauretti et al.[6]; did not observe neurotoxicity in 2 end-stage cancer patients with pain-free intervals that received single-dose treatment with epidural diclofenac sodium. There are no data on outcomes following repeated doses however, prior to intrathecal or epidural use, every drug should be investigated with animal experiments for possible neurotoxic effects [7].

The present experimental study aimed to investigate the possible neurotoxic effects of single and repeated doses of diclofenac sodium administered to rats.

MATERIAL and METHODS

The present study was conducted at the animal laboratory of Gulhane Military Academy of Medicine after obtaining the approval of the Veterinary Faculty of Ankara University Ethics Committee. The study included 24 Sprague -Dawley rats weighing 250-300 g. In compliance with the guidelines for the care and use of laboratory animals, the rats were kept and fed in new cages for 10 days.

Prior to the beginning the study the rats were housed under optimal conditions at 20-22°C and 55% humidity under a 12/12-h day/night cycle, and were fed ad libitum with a standard diet of 20% protein. During the study the same conditions were maintained and each rat was kept in its own polycarbonate cage.

Following a 4-h fasting period each rat received 100 mg kg^{-1} of ketamine HCl and 10 mg kg^{-1} of xylazine HCl via an intraperitoneal route. After achievement of anesthesia and immobilization, intrathecal delivery was performed at the L5-6 intervertebral space with a 0.40×0.50 mm needle, following proper regional shaving and cleaning. The injections were administered (10 µl in volume) after verifying cerebrospinal fluid (CSF) flow with a Hamilton injector (28G sharp tip, SGE, Australia). Following the intrathecal procedure, the rats were monitored with regard to clinical toxicity until they started to move and eat in their cages.

The rats were randomly split into 3 groups. While the rats in group 2 (n=8) received 10 µl (200 µg) of intrathecal diclofenac sodium (Miyadren ampoules, Fako, Istanbul) at 7^{th} day as a single dose, group 1 (n=8) and group 3 (n=8) were administered intrathecal 0.9 % saline (10 µl) and intrathecal diclofenac sodium (10 µl, 200 µg), respectively, each day for 7 days.

All the rats were observed for signs of clinical toxicity and sacrificed under anesthesia via injection of 10% formaldehyde into the ascending aorta for tissue fixation purposes on the 8^{th} day. Spinal cords were explored the following day. Tissue samples acquired from the lumbar region as 1 mm^3 pieces were fixed in 2% glutaraldehyde (pH 7.4) with 0.1 M phosphate buffer for 2 h. At the end of this fixation period the tissues were washed 3 times with buffer and post-fixation was performed via exposure to 1% osmium tetroxide for 1 h. The tissues were then passed through a series of graded alcohol; blocks were then formed by embedding the prepared material using an Araldite CY212 kit. The blocks were polymerized for 48 h at 56°C in a sterilizer. Semi-thin sections were obtained from these blocks and dyed with toluidine blue, and thin sections were acquired from the areas marked under a light microscope. The thin sections were stained with uranyl acetate-lead citrate, and examined and photographed under a Carl Zeiss EM 900 transmission electron microscope (TEM). Histopathologic changes were grades as follows:

0. Normal ultrastructure. 1. Degenerative changes in mitochondria (nuclear content and other organelles are normal). 2. In addition to mitochondrial degeneration, the presence of an irregular structure in rough endoplasmic reticulum (RER), abnormal nuclear content, and extracellular edema. Results between 0 and 1 were scored as 0.5, and results between 1 and 2 were scored as 1.5.

Statistical analysis was carried out using the Kruskal Wallis test. When a difference was present between the groups, the Kruskal Wallis multiple comparison test was performed to determine the group responsible for the difference. $P<0.05$ values were considered statistically significant.

RESULTS

The mobility of the rats in each group was normal at the end of the study, whereas daily mobility was normal in groups 1 and 3. None of the rats were excluded during the study. A statistically significant difference was not observed between the rats in the 3 groups, with regard to body weight before and after the study *(Table 1)* ($P<0.05$).

Table 1. *Body weight of the rats before and after drug administration*
Tablo 1. *Ratların çalışma öncesi ve bitimindeki vücut ağırlıkları*

No	Group	Body Weight on the First Day (g)	Body Weight on the Last Day (g)	P
1	Control	265±18	266±15	>0.05
2	Single-dose	270±15	272±17	>0.05
3	Repeated-dose	266±17	267±15	>0.05

Median scores obtained from histopathologic examinations were 0 (range: 0-0.5) in the group 1 (control), 1 in group 2 (single-dose administered group) (range: 1-1), and 2 in the group 3 (7 days group) (range: 1.5-2). There was a difference between the 3 groups in terms of toxicity scores *(Fig. 1)* (P<0.001). Toxicity in group 2 was greater than that in group 1 *(Fig. 2)* (P=0.002), toxicity in group 3 was greater than that in group 1 *(Fig. 3)* (P=0.003), and toxicity in group 3 was greater than that in group 2 *(Fig. 4)* (P=0.002).

Multipolar neurons in group 1 had cells with a normal structure. The nuclei of the cells were round, centrally

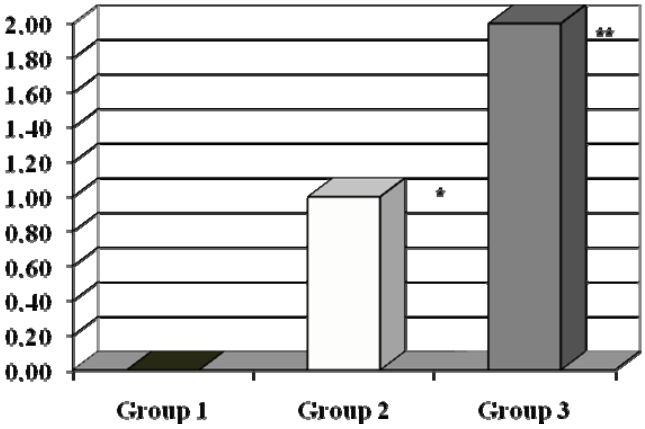

Fig 1. Ultrastructural change score in groups 1, 2, and 3
Şekil I. Grup I, Grup II, Grup III'ün ultrastriktürel değişiklik skorlaması

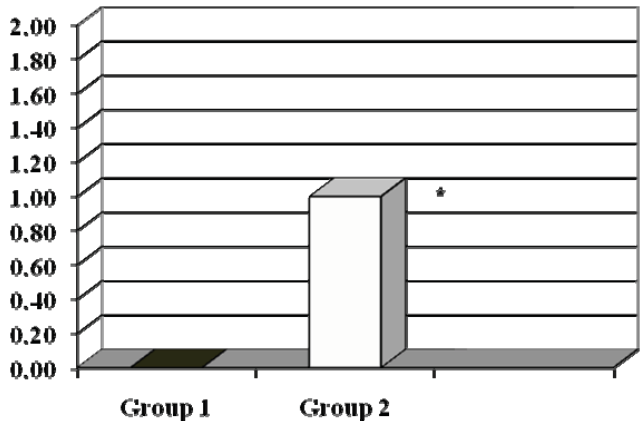

Fig 2. Ultrastructural change score in groups 1 and 2
Şekil 2. Grup I ve Grup II'nin ultrastriktürel değişiklik skorlaması

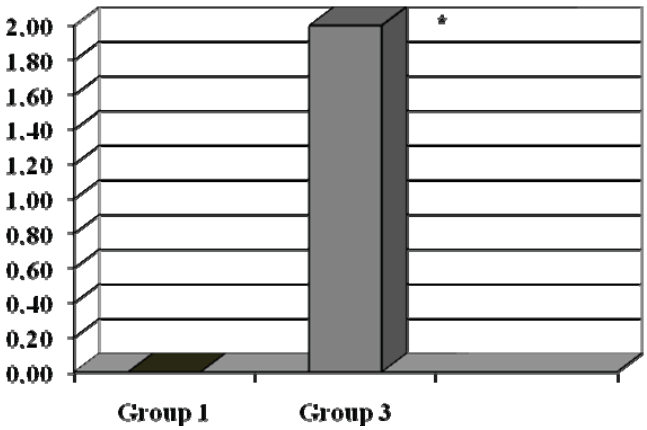

Fig 3. Ultrastructural change score in groups 1 and 3
Şekil 3. Grup I ve Grup III'ün ultrastriktürel değişiklik skorlaması

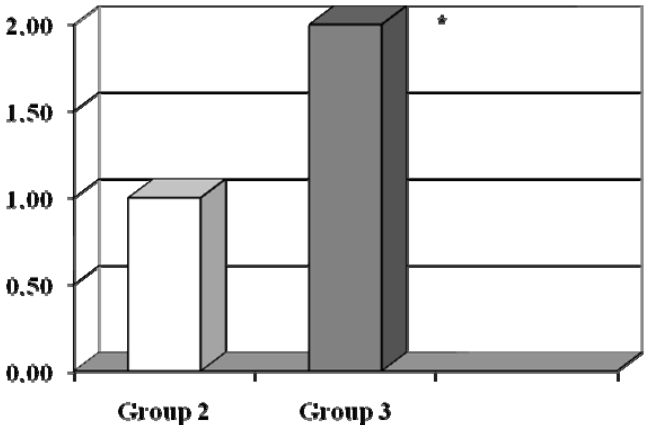

Fig 4. Toxicity results in groups 2 and 3
Şekil 4. Grup II ve Grup III'ün toksisite sonuçları

located, and euchromatic; the nucleoli were prominent. In terms of the cellular cytoplasm, the cisternas of the rough endoplasmic reticulum were fully developed, while the cytoplasm was observed to be electron-dense in relation to ribosome intensity. Mitochondria varied in shape and each had a normal crystal structure *(Fig. 5)*. The general neuron structure in group 2 was similar to that in group 1. Whereas the nuclei and nucleoli were observed have a normal ultrastructure, the cytoplasm was electron-dense. While there were no differences in the cisternas of the rough endoplasmic reticulum, some mitochondria had a reduction in the matrix density; however, in generally, the mitochondria had a normal ultrastructure *(Fig. 6)*.

In group 3 the nuclei had a normal structure, whereas the nucleoli were non-distinctive. The most significant change in the neurons in this group was cellular shrinkage, which led to extracellular edema. The cisternas of the rough endoplasmic reticulum were not fully developed. As some of the mitochondria varied in shape and structure, some exhibited the beginning of cytolysis *(Fig.7)*.

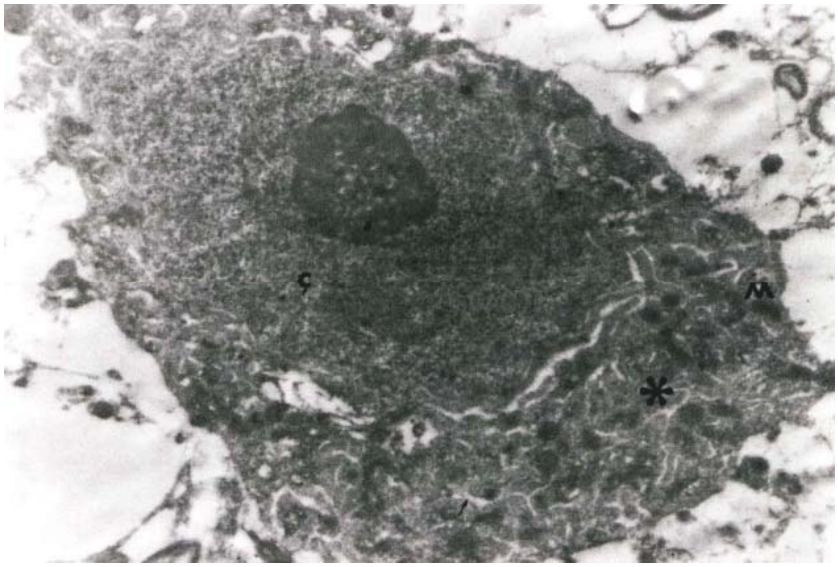

Fig 5. Electron microscopic view of a neuron in group 1. The nucleus (N), RER (↑), mitochondria (M), and electron-dense cytoplasm (*) are normal (uranyl acetate-lead citrate, 4400x)

Şekil 5. Kontrol grubuna ait elektron mikroskobik görünümünde nöron. Çekirdek (Ç), GER (↑), mitokondriyonlar (M) ve elektron yoğun sitoplazma (*) normal görünümde izleniyor (uranil asetat-kurşun sitrat X4400)

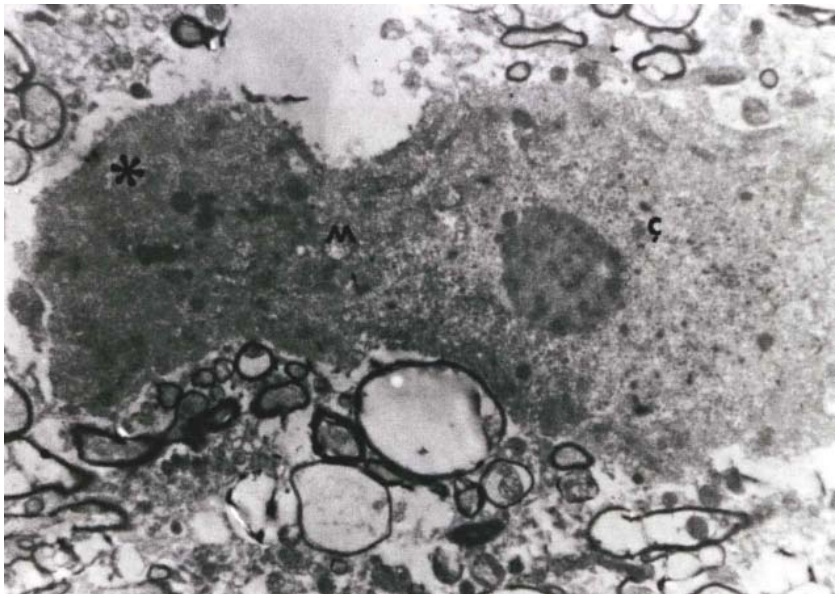

Fig 6. Electron microscopic view of in group 2. The nucleus (N), RER (↑), and electron-dense cytoplasm (*) are normal. A group of mitochondria is exhibiting marked reduction in matrix density

Şekil 6. Tek doz diklofenak uygulanan grubun elektron mikroskobik incelemesi. Nöronlara ait çekirdek (Ç), GER (↑) ve elektron yoğun sitoplazma (*) normal yapıda izleniyor. Mitokondriyonların bir grubunda matriks dansitesinde azalma belirgin (M) (uranil asetat-kurşun sitrat X4400)

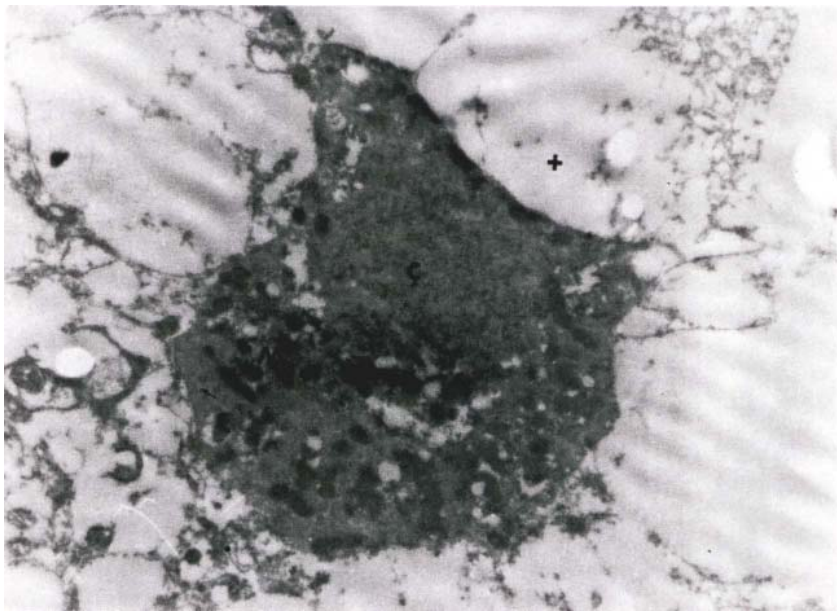

Fig 7. Electron microscopic view of in group 3. The nucleus (N) is normal, whereas a few RER (↑), cytolytic mitochondria, and extracellular edema (+) are distinct (uranyl acetate-lead citrate, 4400x)

Şekil 7. Yedi gün diklofenak uygulanan grubun elektron mikroskobik incelemesi. Çekirdek (Ç) normal yapıda izlenirken, az miktarda GER (↑), kristolizis gözlenen mitokondriyonlar ve ekstrasellüler ödem (+) belirgin (uranil asetat-kurşun sitrat X4400)

DISCUSSION

The present study aimed to determine the signs of neurotoxicity related with time and dose of intrathecal diclofenacsodium. Although the first step in neurotoxicity research is light microscopy, one can obtain more detailed data with an electron microscope, which has higher sensitivity. As such, we conducted histopathologic examinations via electron microscopy.

There are no conclusive data verifying that the presence of microscopic changes in the structures of myelin, the neuron body, or axonal extensions are sufficient [8]. In the current study we scored alterations in the nerve cell organelles using electron microscopy and investigated possible neurotoxicity. It is yet unknown whether or not studies focusing on neurotransmitters and receptors are necessary [8].

Despite data obtained from studies on the effects of lead and mercury, as well as from those investigating the outcomes of chronic spinal exposure to local anesthetics, the most appropriate animal model for neurotoxicity studies or research on the specific lesions in the spinal cord that occur due to neurotoxicity are not yet known [8]. Nonetheless, most of the agents that do not cause neurotoxicity in rats have been used in humans for many years. Most reports indicate that rats are appropriate for toxicity studies. Yet, neurotoxicity studies on animals are associated with many technical problems. The sensitivity of the model used in neurotoxicity studies bears some importance as well. Rats are the most commonly used animals for demonstrating the toxic effects of various agents. Many studies conducted with rats have shown that even a single dose of some agents could lead to irreversible motor dysfunction and histopathologic damage. For example, Gauman et al.[9] reported that intrathecal single-dose somatostatin caused motor paralysis, as well as inflammation and focal demyelinization in the spinal cord. As such, we conducted the present study with rats.

Direct neurotoxic effects are thought to be primarily associated with the concentration of the agent used [10]. Delivery of drugs at high concentrations has been shown to cause irreversible nerve conduction block in in vitro studies on axons [11,12]. In 1991 Malinovsky et al.[13] studied the neurotoxicity of ketamine and midazolam in rabbits. Histopathologic changes reported in previous studies conducted with ketamine are thought to have occurred due high ketamine concentrations or technical difficulties encountered during dural puncture. Midazolam was reported to cause significant changes in both the spinal cord and blood-brain barrier levels [13].

Takenami et al.[14] studied the effects of varying concentrations of intrathecal tetracaine on rat spinal cords, and observed myelin destruction and axonal degeneration, which were dose dependent [14]. In the present study we similarly observed an increase in neurotoxicity that was associated with the duration of diclofenac sodium administration.

Abram et al.[15] investigated the neurotoxic effects of intrathecal corticosteroids with the help of electron microscopy. Some preparations elicited edema that caused the vascular wall to separate from the surrounding tissues. Based on electron microscopic examination, it was suggested that those dark-colored neurons were formed as a result of the accumulation of intracytoplasmic dark granules and filaments [15]. In the current study electron microscopy revealed extracellular edema associated with cell shrinkage in neurons.

While epinephrine and phenylephrine can be used for prolonging the duration of spinal anesthesia, glucose can be added to increase baricity, and antioxidant or preservative agents can be used for preservative purposes. Neurotoxicity can be influenced by those additional agents, as well as by the drug itself. Animal studies have shown that there are no neurotoxic effects associated with epinephrine, whereas phenylephrine was reported to elevate the risk of neurologic symptoms when used in combination with tetracaine [16]. Hyperglycemia can progressively damage nerve fibers, depending on the severity and duration; however, glucose concentrations below 7.5% can be delivered safely via the spinal route in order to increase baricity [17].The neurotoxic effects of ethylene diamine tetra-acetate (EDTA) and sodium bisulfide have been histopathologically shown [16]. Among such preservatives as parabens, chlorobutanol, benzethonium chloride, and benzyl alcohol, parabens are reported to be the safest [18,19]. The diclofenac sodium preparation used in the present study was comprised of mannitol, sodium metabisulfite, benzyl alcohol, propylene glycol, and sodium hydroxide, and the neurotoxicity observed in the present study may have been due to those substances. It is important that agents delivered into the subarachnoidal space be diluted adequately with cerebrospinal fluid, as the main factors affecting neurotoxicity are the concentration of agents on the surface of the spinal cord and the duration the spinal cord is exposed to those agents. Acute elevation in cerebrospinal fluid volume due to the volume of the delivered agents may lead to decreases in spinal blood flow and development of neurologic deficits; therefore, we administered diclofenac sodium with 10 µl of saline, which was previously shown to cause no increase in intracranial pressure [9,15].

Lauretti et al.[6] did not observe any signs suggestive of neurotoxicity following single-dose diclofenac sodium treatment in 2 end-stage cancer patients; however, no data were provided concerning repeated doses. Lauretti et al.[6] underscored the fact that morphological examination alone is not adequate for studying neurotoxicity, and

that it should be supported by functional analysis and examination of the interactions of spinal cord blood flow [6].

In the present study mild signs of toxicity were noted in group 2, versus more severe signs in group 3. Rats in group 3 had clinically normal vital signs and neurologic deficits were not observed; however, at the cellular level there were ultrastructural changes in the neurons-evidence of neurotoxicity. We conclude that diclofenac sodium could lead to neurotoxicity when administered intrathecally and cause some complications in clinical use. We think that when delivering a drug via the intrathecal route, in terms of neurotoxicity, one should not only consider the active ingredient, but also the excipients.

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