

Investigation of Neuroprotective Effects of Cyclooxygenase Inhibitors in the 6-Hydroxydopamine Induced Rat Parkinson Model

Siklooksijenaz İnhibitörlerinin 6-Hidroksidopamin ile Oluşturulan Deneysel Parkinson Modelinde Nöroprotektif Etkilerinin Araştırılması

ABSTRACT

AIM: Recent experimental and clinical studies on Parkinson's disease point out the pivotal role of inflammation in the pathogenesis of neurodegeneration and the possible positive effects of nonsteroidal anti-inflammatory drug therapies. Our aim in this study was to investigate the preventive effects of nonsteroidal anti-inflammatory drugs in the 6-hydroxydopamine (6-OHDA) induced rat model of Parkinson's disease.

MATERIAL and METHODS: Twenty-one female Wistar-Albino rats (200-250g) were used in this study. The rats were divided in three groups: Saline group (n: 7, 2 ml), Acetylsalicylic acid group (n: 7, 100 mg/kg), and Meloxicam group (n: 7, 50 mg/kg). An hour after administration, the rats received a unilateral intranigral injection of 6-OHDA to produce the Parkinson model lesion. Rotational tests were performed two weeks later as follow-up. Immunohistochemical tests were performed in all groups to determine the severity of the lesion in the substantia nigra.

RESULTS: Administration of drugs an hour before the lesions were created did not protect the degeneration of dopaminergic neurons in the substantia nigra.

CONCLUSION: Oral usage of low repeated doses of nonsteroidal anti-inflammatory drugs may possibly slow down the progression of the disease.

KEY WORDS: 6-OHDA, NSAID, Parkinson model, Rat

ÖZ

AMAÇ: Parkinson Hastalığı, bazal ganglionların substantia nigra bölgesindeki dopaminerjik nöronların ölümüyle karakterize dejeneratif bir bozukluktur. Sayıları giderek artan deneysel ve klinik çalışmalar, nörodejeneratif bu hastalığın patogeneğinde inflamasyonun rolü ve non-steroid antiinflatuar ilaçların koruyucu etkilerinin rolünü araştırmayı amaçlamaktadır. Bu amaçla NSAİİ'lerin 6-OHDA ile oluşturulmuş sıçan Parkinson modelindeki koruyucu etkileri araştırıldı.

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YÖNTEM ve GEREÇ: Çalışmada, 200-250 g ağırlığında 21 adet dişi Wistar-Albino cinsi sıçan kullanıldı. Rastgele gruplara ayrılan sıçanlara enteral yolla serum fizyolojik, asetil salisilik asit ve meloksikam uygulandı. Bu uygulamadan bir saat sonra sıçanlara Parkinson modelini oluşturmak için intranigral olarak tek taraflı 6-hidroksidopamin enjeksiyonu gerçekleştirildi. Bu enjeksiyondan iki hafta sonra da sıçanlarda Parkinson modeli oluşup oluşmadığını gözlemek için dönme testleri uygulandı. Substantia nigra bölgesindeki lezyonun gösterilebilmesi için immünohistokimyasal testler kullanıldı.

BULGULAR: Çalışmamızda, bu ilaçların lezyondan bir saat önce uygulanması, substantia nigra bölgesindeki dopaminerjik nöronları dejenerasyondan korumamıştır.

SONUÇ: Bu çalışmada, NSAİİ'lerin akut tek doz kullanılması dejenerasyonu önlemese bile, oral yolla uzun süreli tekrarlayan kullanımları bu hastalığın ilerlemesini yavaşlatabilir.

ANAHTAR SOZCÜKLER: 6-OHDA, NSAİİ, Parkinson modeli, Sıçan

INTRODUCTION

Parkinson's disease (PD) is a degenerative disorder of the basal ganglia characterized by selective and progressive degeneration of dopaminergic neurons settled in the pars compacta of substantia nigra (SNpc). This degeneration results in a decreased projection of the nigrostriatal dopaminergic system that clinically manifests with bradykinesia, muscular rigidity, resting tremor and postural instability.

Although many investigations have focused on the dopaminergic neuronal degeneration, the activation of the glial cells might also play an important role in the initiation and progression of cell death in PD (8,17).

6-hydroxydopamine (6-OHDA) inhibits complex I in mitochondria and increases intracellular free radical generation. Toxic effects of 6-OHDA can contribute to the degeneration of dopamine-containing neurons by releasing superoxide and cytokines from microglia. These cytokines activate iNOS and COX-2 in microglia that produce PGs. PGs can exert a direct effect to induce toxic events in dopaminergic neurons (6).

Inflammation is a prominent feature of many degenerative diseases of the central nervous system such as PD. Local brain inflammatory reaction depends upon the synthesis of inflammatory components by local neurons and glia, particularly microglia, instead of antibodies and significant involvement of T cells (2,15).

Microglia are sensitive to changes in the microenvironment and readily become activated in response to extraordinary circumstances like brain infection or inflammation. They are one of the major cellular substrates of cytotoxic mechanisms (12).

Due to the rich environment of microglia and

neuronal sensibility, the substantia nigra is prone to attacks imposed by factors produced by activated microglia. Therefore it is suggested that microglia may play an important role in PD so inhibition of the inflammatory response might result in a sparing of neuronal viability.

Cyclooxygenase (COX) is the enzyme that catalyses the first two steps in the biosynthesis of PGs from the substrate AA (29). There are at least two isozymes of COX, COX 1 and COX 2. Both isozymes catalyze the same two reactions: oxygenation of arachidonate to yield PGG₂ and reduction of PGG₂ to PGH₂. COX 1 is expressed constitutively and is present in most tissues. COX 2 is induced by inflammatory stimuli, such as cytokines (25,28). Nonsteroidal anti-inflammatory drugs (NSAIDs) produce therapeutic effects through inhibition of COX.

Acetylsalicylic acid shows its neuroprotective effects via scavenging hydroxyl radicals (5), nitric oxide synthase inhibition (19,23), and the inhibition of the activation of the transcription factor NF- κ B, (7,10) in animal models of PD.

There is an up-regulation of COX-2 in nigrostriatal dopaminergic neurons in lesioning both with 6-OHDA or MPTP and human PD samples (22,27). COX-2 activity is responsible for many of the cytotoxic effects of inflammation which accounts for the large amounts of PGs seen in inflammation sites (24). Hence, COX-2 inhibitors such as meloxicam, celecoxib are used to acquire pronounced protection (22). Meloxicam is a preferred choice as a preferentially selective COX-2 inhibitor because it has significantly fewer gastrointestinal side effects than the standard NSAIDs (29,30).

In our study we aimed to prevent dopaminergic cell degeneration in an experimental PD model by

using a high dose therapy of ASA and meloxicam enterally.

MATERIALS and METHODS

Figure 1 shows a schematic diagram of the investigation.

Animals

The study protocol was approved by the local Animal Care and Use Committee. Twenty-one adult female Wistar albino rats weighing 200–250 g were used. The rats were obtained from the Uludag University Experimental Animals Breeding and Research Center, and were housed two per cage in a temperature-controlled environment (21°C) on a 12:12-hr light/dark cycle. Food and water were offered ad libitum.

The animals were randomly divided into three groups. Group I rats (n: 7) received 2 ml saline by nasogastric cannula before being lesioned with 6-OHDA. Group II rats (n: 7) received 100 mg/kg acetylsalicylic acid 1 hour before lesioning with 6-OHDA. Group III rats (n: 7) received 50 mg/kg meloxicam 1 hour before lesioning with 6-OHDA. Acetylsalicylic acid and meloxicam were prepared as a suspension in saline just before the administration and were given by nasogastric cannula into the stomach after anesthetization of rats with sodium pentobarbital (30 mg/kg i.p.).

Lesion Procedure

One hour after administration of the drugs (acetylsalicylic acid, meloxicam, and saline), each rat was placed in a stereotaxic frame (ASI Instr., USA). The coordinates of the substantia nigra were located (5.0 mm posterior and 2.0 mm lateral to the bregma). Next, 4 µl (8 µg) of 6-OHDA-hydrobromide was drawn into a 10-µl Hamilton syringe and infused into the substantia nigra (7.4 mm below the dural surface at the burr-hole site).

Rotational Testing

Two weeks after the lesion surgery, each animal in Groups I, II and III underwent rotational testing to determine the extent of the lesion based on the severity of motor behavioral disorder. The tests were performed with amphetamine (5 mg/kg i.p.), and apomorphine (0.1 mg/kg) in a Rota-Count® apparatus (Columbus Inst., Ohio, USA). A record was made of the number of full turns (360°) to the ipsilateral and to the contralateral sides of the lesion that each rat made during 1 hr.

Immunohistochemistry

Immunohistochemical tests were performed to determine if the establishment of the lesion in the substantia nigra was achieved. Briefly, following the final rotational tests, the rats in all three groups were decapitated; brains were removed and fixed in 10% formaldehyde for 24 hrs and 50 µm-thick serial coronal sections were cut with a vibrotome. After incubating in blocking buffer to block non-specific binding, the sections were incubated with rabbit anti-tyrosine hydroxylase antibody (1:3000; AB152, Chemicon Inc., Temecula, CA, USA) for 48 hr at room temperature. The sections were then incubated in appropriate biotinized secondary antibody (1:200; Jackson Immunosearch Labs, West Grove, PA, USA) followed by incubation in avidin-biotin complex (ABC Kit, Vector Laboratories Inc., Burlington, CA, USA). Sections were then exposed to 0.05% diaminobenzidine solution (25 mg diaminobenzidine and 5 ml H₂O₂ in 100 ml Tris-HCl buffer) and mounted onto slides.

To evaluate each rat’s substantia nigra, we examined serial sections between –4.80 mm and –5.80 mm relative to the bregma in rostrocaudal direction, according to The Rat Brain Atlas by Paxinos and Watson (18). Five sections per animal

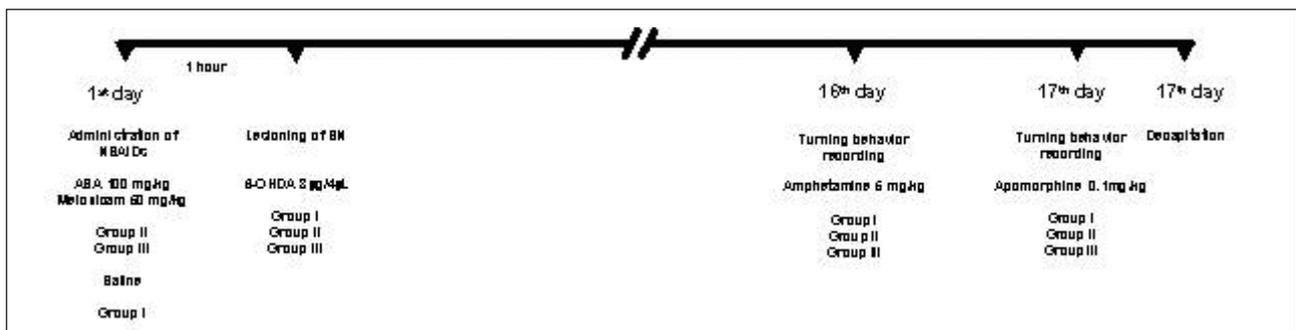


Figure 1: Schematic diagram of the study.

were analyzed for the survival of dopaminergic neurons using an Olympus BX-50 photomicroscope (Olympus Optical Corp., Tokyo, Japan). The number of dopaminergic neurons was counted by two observers who were blind to the experiments. Data were expressed as mean ± SEM and were subjected to ANOVA. $p < 0.05$ was considered statistically significant.

Statistics

The group results for rotational behavior testing are expressed as mean ± SD. For statistical evaluation, the group data were subjected to one-way analysis of variance (ANOVA) and the Dunn’s multiple comparisons test. p values < 0.05 were considered to reflect statistical significance.

RESULTS

Rotational Behavior

Table I represents the full turn (360°) counts for one hour for group I (saline), group II (acetylsalicylic acid), and group III (meloxicam) after amphetamine and apomorphine administration at the post-lesioning stages. Figure 2 shows the results after amphetamine, and figure 3 shows the results after apomorphine administration at the post-lesioning stages for all groups. Positive values represent turning contralaterally to the lesion, and negative values represent turning ipsilaterally to the lesion. Following the lesioning procedure all rats developed PD.

In Group I with amphetamine, the mean full-turn count was 346.9 ± 39.7 after lesioning with 6-OHDA. This value was 315.4 ± 38.7 in Group II, and the corresponding finding in Group III was 261.7 ± 65.6 with amphetamine at the post-lesioning stage with 6-OHDA. At the post-lesioning stage, there were no statistically significant differences when all the groups were compared ($p > 0.05$) (Table I, Figure 2). The testing with apomorphine was performed 24 hours after the testing with amphetamine. When we tested after apomorphine administration, the mean full-turn count in Group I

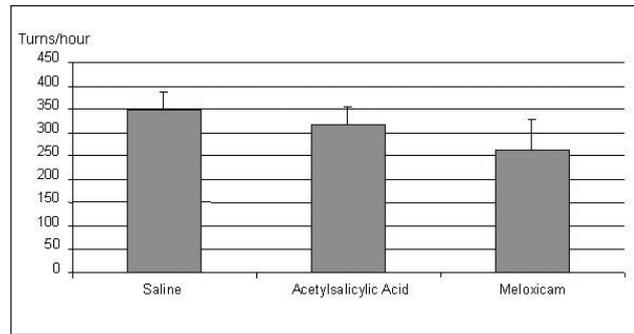


Figure 2: Turning values induced by amphetamine (Mean ± SD) after lesioning with 6-OHDA.

was -180.1 ± 58.0 after lesioning with 6-OHDA. In Group II it was -258.9 ± 81.2 , and in Group III the value was -158.6 ± 29.8 . At the post-lesioning stage, there was no statistically significant difference between the groups similar to the amphetamine testing ($p > 0.05$) (Table I, Figure 3)

Immunohistochemistry Findings

Immunohistochemical staining of brain sections from the control group (no lesioning) revealed multipolar cells containing tyrosine hydroxylase (TH) in the substantia nigra and the ventral tegmental areas (Figure 4A,B). 6-OHDA administration led to a significant decrease in the number of the TH immunoreactive neurons in the lesioned SNpc (Figure 4C). However, on the intact side, the density of TH immunoreactive cells was

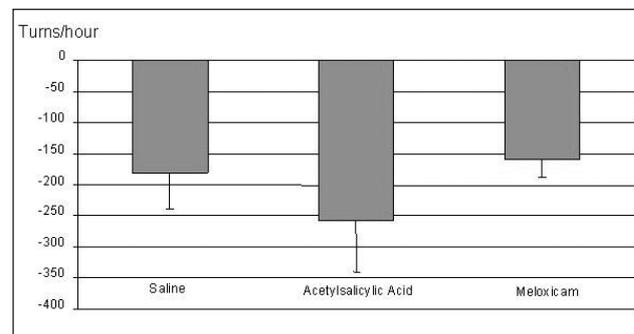


Figure 3: Turning values induced by apomorphine (Mean ± SD) after lesioning with 6-OHDA.

Table I: Turning values in all three groups (full turn /hour, mean ± SEM)

	Saline (n:7)	Acetylsalicylic Acid (n:7)	Meloxicam (n:7)
Amphetamine	346,9 ± 39,7	315,4 ± 38,7	261,7 ± 65,6
Apomorphine	- 180,1 ± 58,0	- 258,9 ± 81,2	- 158,6 ± 29,8

+ values represent turning away from the lesioned hemisphere.
 - values represent turning to the lesioned hemisphere.

similar to that of the control group (Figure 4D). The ventral tegmental area on the lesioned side showed TH staining (Figure 4C). High dosages of ASA (Figure 4E,F) and meloxicam (Figure 4G,H) produced no attenuation of 6-OHDA induced loss of TH-positive cells. Acetylsalicylic acid and meloxicam administration did not increase the quantity of TH-positive neurons in substantia nigra compared to the control group (Table II, Figure 5).

DISCUSSION

Since the middle of 1990's, many groups have been interested in the use of NSAIDs in the animal model of Parkinson disease. Some of them have been in vivo animal models.

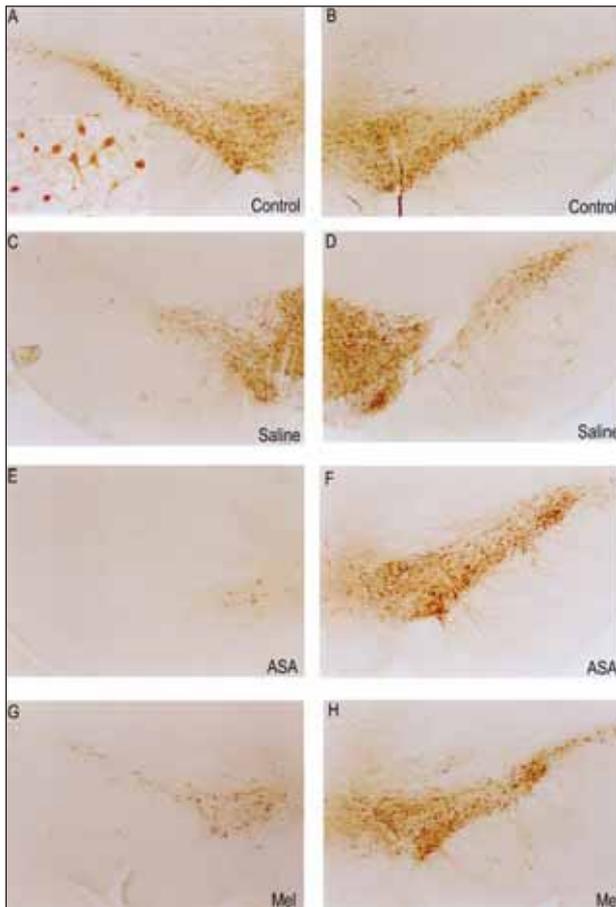


Figure 4: Photomicrographs of SNc sections, stained with an antibody against TH; of control group without drug and 6-OHDA (A, B) and treated saline (C, D), ASA (100 mg/kg) (E; F) or meloxicam (50 mg/kg) (G, H) one hour after a single administration of 6-OHDA. Inlet demonstrating the normal appearance of TH-positive neurons (x40). C, E, and G are from the lesioned side while D, F, and H are from the intact side of the same animal (x4).

Table II: The number of TH-positive neurons in substantia nigra. Data are summarized as the mean ± SEM number of neurons per animal. The p value is 0.43.

Groups	Mean number of TH-positive neurons counted per animal
Saline (n:7)	135.86 ± 20.44
ASA (n:7)	91.29 ± 25.14
Mel (n:7)	98.43 ± 14.33

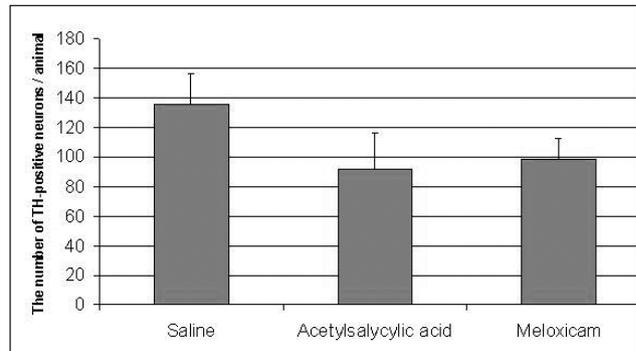


Figure 5: The effects of acetyl salicylic acid (100mg/kg) and meloxicam (50mg/kg) or saline treatment on the 6-OHDA induced decrease of TH-positive cell count. Data is mean ± SEM (n = 7).

Aubin et al. administered ASA and salicylate derivatives intraperitoneally at different times before and after a single usage of MPTP (1). They concluded that significant protection was observed between 2 hours before and after MPTP administration and maximal protection was observed when ASA was given concomitantly or one hour after MPTP administration. They also concluded that the neuroprotective effects of salicylate derivatives could perhaps be related to hydroxyl radical scavenging and speculated that the protective effects of aspirin are unlikely to be related to cyclooxygenase inhibition as paracetamol, diclofenac, ibuprofen and indomethacin were ineffective.

Ferger et al. used C 57BL/6 mice and were treated with a single dose of sodium salicylate (50 mg/kg or 100 mg/kg ip) or saline immediately before injection of MPTP (30 mg/kg or 40 mg/kg sc) or saline (5). They concluded that the MPTP-induced loss of tyrosine hydroxylase immunoreactivity in nigral cell bodies was nearly completely prevented by the higher dose of sodium salicylate. The protective effects of salicylate against reversible or irreversible

impairments in dopaminergic neurotransmission after MPTP treatment may be related to its radical scavenging properties. Ferger and colleagues pointed out that the neuroprotective properties of salicylic acid are based on its effect as a hydroxyl radicals scavenger rather than other mechanisms (5).

Mohanakumar et al. used mice treated with MPTP and suggest that salicylic acid acts as a free radical scavenger in the brain that indicates its effectiveness as a valuable neuroprotectant (16). Also Sairam et al. found the salicylic acid to be neuroprotective in the MPP rat model (21). There are some other experiments that found ASA to be neuroprotective in the ROS scavenging mechanism (3,4,13,14).

On the other hand, there are controversial results about especially COX-2 inhibitors about neuroprotection. For example Teismann et al. used different doses of NSAIDs (26). They had indicated that 100 mg/kg/ip ASA and 50 mg/kg/ip meloxicam significantly attenuated the decrease of locomotor activity, and nigral cell loss was almost completely prevented by these drugs. They concluded that the inhibition of COX-1/COX-2 by ASA or preferentially COX-2 by meloxicam provided significant neuroprotection against MPTP toxicity on the dopaminergic neurons by intraperitoneal administration just before MPTP administration. Teismann et al. showed that the MPTP-induced decrease of TH-IR as well as the loss of nigral neurons was nearly completely prevented by acetylsalicylic acid and meloxicam (26) and stated that the inhibition of either COX-1/COX-2 by acetylsalicylic acid or preferentially COX-2 by meloxicam provided clear neuroprotection against MPTP toxicity at the the striatal and nigral levels.

In the experiment of Kurkowska-Jastrzebska et al., indomethacin protected neurons against toxic damage caused by MPTP in a mice model of Parkinson's disease (11). Klivenyi et al. found that administration of rofecoxib with creatine produced significant additive neuroprotective effects against dopamine depletions (9).

Sanchez-Pernaute et al. had used celecoxib 14 or 21 days enterally and investigated its protective effect on dopaminergic neurons (22). They had used 6-OHDA and had administered the neurotoxin intrastrially so progression of dopaminergic neural degeneration occurred slowly over the course of two weeks. Twelve days after the 6-OHDA lesion, they

could not find any difference between the groups in dopaminergic cell or fiber loss although the microglial cell density and activation was markedly reduced in animals receiving celecoxib. They could not find the same degree of degeneration in the celecoxib group between 12 and 21 days although there was significant cell loss in the vehicle group. They noted that this effect could have been due to the inhibition of COX-2 directly or through inhibition of microglia activation to prevent or slow down dopaminergic cell degeneration.

However Sairam et al. found that treatment with the selective COX-2 inhibitor celecoxib exacerbated MPP+-induced decrease in dopamine levels (21). They concluded that the failure of celecoxib to render protection in animals against MPP+-induced dopamine depletion indicates absence of prostaglandin involvement in MPP+ action. These results also suggest that the neuroprotective ability of salicylic acid is independent of prostaglandin mediation and COX-2 inhibition has similar effects (20). They have demonstrated that COX-2 activity inhibition by rofecoxib, started 1 day after the injury, had no neuroprotective effect. Their study suggested that COX-2 does not contribute to neuron death following MPTP administration and that the inhibition of COX-2 activity was not beneficial to neurons injured by MPTP.

Teismann et al. report that studies with MPTP show that inhibition and ablation of COX-2 markedly reduce the deleterious effects of the toxin on the nigrostriatal pathway (27). They have speculated that COX-2 expression is also pathogenic in PD. However enteral administration of high dose acetylsalicylic acid (ASA) and meloxicam an hour before lesioning with 6-OHDA did not prevent the degeneration of dopaminergic neurons in substantia nigra. Although intraperitoneal usage of these drugs showed neuroprotective effects, we could not observe the same effects with enteral usage in our Parkinson model. We decided to use this model of PD in the rat to evaluate the protective effects of aspirin and meloxicam as a preliminary study.

We wondered if we could protect dopaminergic neurons from neurotoxicity by using a high dose of meloxicam and nonselective COX inhibitor ASA enterally as a single treatment prior to giving the neurotoxin. We speculate that single high dose could not reach the protective level as the chronic administration of Sanchez-Pernaute et al.'s study (22). In our study, the protective effects of NSAIDs

did not occur with this dosage so we found diffuse degeneration of dopaminergic neurons in substantia nigra. We can speculate that even if we could inhibit the COX with a single high dose of these drugs enterally, there would be no effect on the other mechanisms that lead to neuronal death according to these results.

As a result, we suggest that oral usage of low repeated doses of NSAIDs may possibly slow down the progression of the disease. Different doses and different usage times are needed to observe the protective effects of these drugs enterally. Further investigations are needed to prove this hypothesis.

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