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Nociceptive improvements and kynurenine pathway alterations with diclofenac treatment in a rat model of neuropathic pain created by partial sciatic nerve ligation

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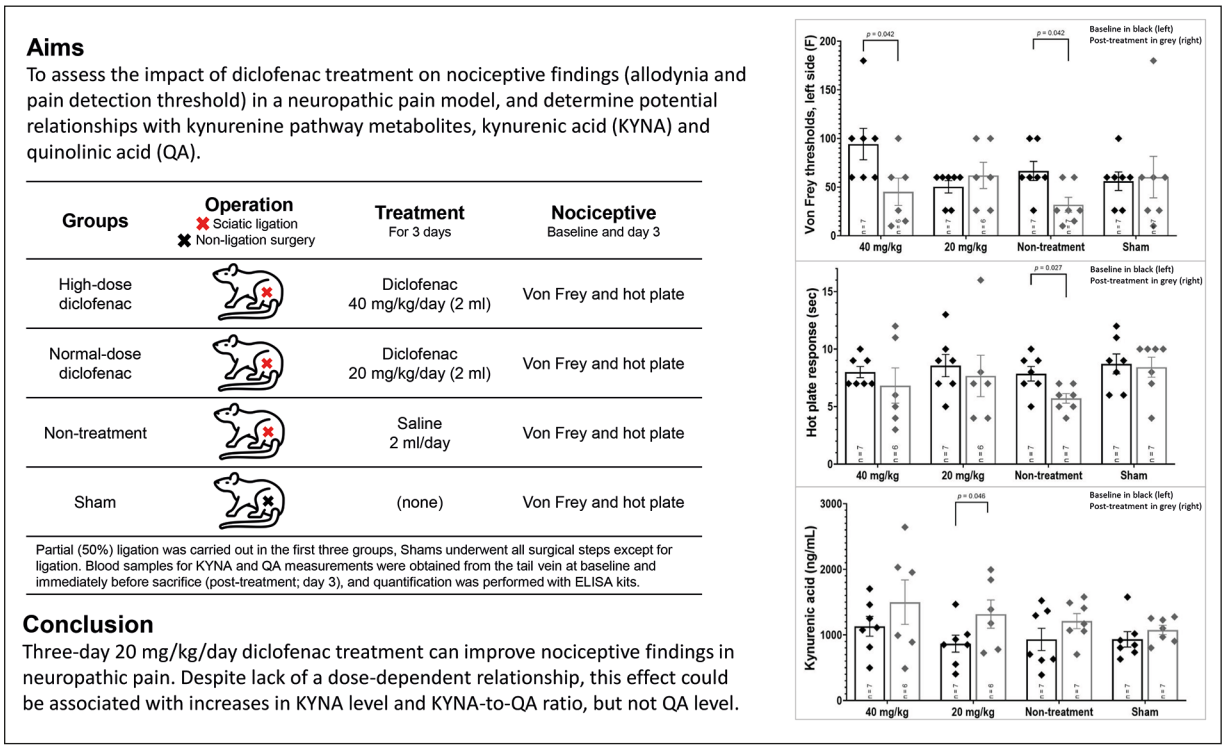
Abstract. – OBJECTIVE: Neuropathic pain is regulated by several metabolites of the kynurenine pathway (KYNA-kynurenic acid, and QA-quinolinic acid). Diclofenac exerts analgesic and anti-hyperalgesic effects and also alters KYNA levels, indicating a potential for therapy. We aimed to assess the nociceptive effects of different doses of diclofenac treatment in a rat model of neuropathic pain, and to determine potential relationships with KYNA and QA levels (Graphical Abstract).

MATERIALS AND METHODS: Twenty-eight Sprague-Dawley rats were divided into four groups: 40 mg/kg/day diclofenac (high-dose), 20 mg/kg/day diclofenac (normal-dose), non-treatment, and sham. Except for the sham group, the others underwent partial sciatic nerve ligation (left). Baseline (day 0) and post-treatment (day 3) KYNA and QA levels were measured. Allodynia and pain detection were assessed with the von Frey and hot plate tests.

RESULTS: Baseline findings were similar in all groups. Compared to baseline, the non-treatment group had significantly worse allodynia on day 3. Baseline and post-treatment von Frey results (left) remained similar in the nor-



Graphical Abstract. Study groups, results concerning the most striking findings, and conclusions drawn from the study with respect to design and analyses.

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mal-dose diclofenac group (p=0.336); however, this benefit was not observed in the high-dose group. Relative to baseline, normal-dose diclofenac recipients had significantly higher KYNA concentration (p=0.046) and KYNA-to-QA ratio (p=0.028) on day 3.

CONCLUSIONS: Our results show that 3-day therapy with 20 mg/kg/day diclofenac can improve nociceptive findings in neuropathic pain, and that this effect may be associated with increased KYNA or KYNA-to-QA ratio. The lack of dose-dependent effects may be associated with potential adverse influences of exceedingly high diclofenac dosage.

Key Words:
Neuralgia, Diclofenac, Kynurenic acid, Quinolinic acid, Allodynia.

Introduction

Neuropathic pain is encountered in 3-12% of the population and represents an important cause of chronic pain[1]. It can develop through unclarified mechanisms after disease or trauma affecting the central or peripheral nervous systems[2]. Various likely contributors to neuropathic pain have been established[3]; however, N-methyl-D-aspartate (NMDA) receptors have long been studied for their role in neuropathic pain and it appears that perpetual activation of NMDA receptors may cause hyperalgesia and allodynia[4], and beneficial effects with blockade of NMDA receptors have been reported[4,5] in patients with neuropathic pain.

The kynurenine pathway metabolizes tryptophan into kynurenine, kynurenic acid (KYNA), and other compounds including quinolinic acid (QA), ultimately producing NAD+[6]. Dysfunctions in the pathway have been associated with problems in serotonin metabolism and neuroplasticity, as well as neurodegenerative diseases and aging[7-9]. NMDA receptors can be modulated by KYNA (antagonist) and QA (agonist), possibly leading to alleviation of pain through KYNA increase and worse pain through QA increase[10,11]. Indeed, studies[12-15] in murine models have shown relationships between pathway metabolism and the nociceptive findings associated with neuropathic pain (allodynia, tactile hypersensitivity, etc.), presumably through NMDA receptor antagonism. Also, administration of KYNA at very low concentrations was shown to decrease glutamate levels in the brain – another route by which antagonistic effects on NMDA receptors may occur[16].

In addition to being a cyclooxygenase (COX) 1 and 2 inhibitor, diclofenac exerts analgesic and anti-hyperalgesic effects through central mechanisms[17,18]. Furthermore, it has been demonstrated to stimulate KYNA synthesis under specific conditions[19], indicating potential as a therapeutic for neuropathic pain[11]. However, currently there is limited evidence showing that neuropathic pain is directly associated with plasma levels of the NMDA receptor-regulating metabolites of the kynurenine pathway (i.e., KYNA and QA). There is a need for further studies to investigate possible associations with nociceptive findings in neuropathic pain and the levels of kynurenine pathway metabolites. Therefore, we planned this experimental animal study to determine the effect of diclofenac therapy on nociceptive findings associated with neuropathic pain, and to assess whether potential changes in nociceptive findings were associated with plasma levels of KYNA and/or QA.

Materials and Methods

Study Design and Sample Size

The experimental protocol of this study was approved by the Kobay Animal Studies Laboratory Ethics Committee (approval code: 541, date: April 20, 2021), and 28 Sprague-Dawley adult rats (250-300 g) were bought through the same laboratory and were acclimatized to their cages for 2 weeks before study initiation. Experiments were carried out in October 2021 at Kobay Animal Studies Laboratory, Ankara, Turkey. Rats were housed with simulation of 12-hour day/night cycles in sufficiently large cages maintained at 22±2°C and 45-65% humidity, with ad *libitum* access to tap water and standard rodent chow, both before and after interventions.

Since there is no reliable data concerning plasma levels of KYNA or QA in rats with neuronal injury or neuropathic pain, we employed the Resource Equation method to determine adequate sample size that would enable comparison of different treatment doses. The degree of freedom (dF) achieved for analysis of variance (ANOVA) was utilized to estimate necessary sample size[20]. Excluding the sham group, we found that the maximum number of rats that could be included to achieve a dF value between 10-20 (target value) was 7 in each group (dF=total animal count - group count). With 7 rats, the dF value was calculated as 18 (7×3-3), and therefore, we included 7 rats in each group, resulting in a total of 28 rats with the sham group.

Experimental Groups

The experimental groups and treatments were as follows. Group 1 (high-dose diclofenac): partial (50%) sciatic nerve ligation to induce neuropathic pain and intraperitoneal high-dose (40 mg/kg/day) diclofenac therapy (2 ml) for 3 days. Group 2 (normal-dose diclofenac): partial (50%) sciatic nerve ligation to induce neuropathic pain and intraperitoneal normal-dose (20 mg/kg/day) diclofenac therapy (2 ml) for 3 days. Group 3 (non-treatment): partial (50%) sciatic nerve ligation to induce neuropathic pain and 2 ml/day of isotonic saline (0.9% NaCl) for 3 days. Group 4 (sham group): non-ligation surgery without diclofenac or saline treatment. All rats were sacrificed *via* CO_2 exposure 72 hours after surgery.

Nociceptive Assessment

All animals underwent the von Frey and hot plate tests to assess mechanical allodynia and pain detection threshold, at baseline (day 0) and after treatment (day 3)[21]. The von Frey test was applied after 15 minutes of habituation on a caged floor and according to the "percent response" method with 5 tests for each filament (defined as trials). Starting from the filament applying 1 gram of force (F), trials (5 tests for each filament) were performed successively by increasing filament strength after each trial (1, 1.4, 2, 4, 6, 8, 10, 15, 26, 60, 100, 180 and 300 gram filaments). We allowed animals at least 5 minutes between each trial to prevent learned responses. After a trial with 100% response (5 positives out of the 5 tests in the trial) was recorded, we performed a further trial with the previous (weaker) filament to assess whether 100% response could be observed. This ascending / descending trial process was continued until the lowest force threshold yielding 100% response was determined.

Hot plate tests were applied in a routine manner with the plate set to 55°C. Time (seconds) until front/hind paw licking, paw withdrawal or jumping behaviors occurred were observed for a maximum of 15 seconds. The results of tests in which rats did not demonstrate characteristic responses within this time limit were recorded as 16 seconds (n=1).

Surgical Procedure

The neuropathic pain model was created with partial sciatic nerve ligation, as described by Seltzer et al[22], and all surgeries were carried out by the same two researchers (S. Debbag and A. Yalcinkaya). Briefly, the rats were anesthetized with 50 mg/kg pentobarbital, the left gluteal region was shaved to obtain a 6×6 cm surgical area which was washed with copious amount of povidone iodine, and rats were put into the left decubitus position before surgery. Standard incisions were performed until the biceps femoris muscle was visualized. The left sciatic nerve was explored after incision of the biceps femoris, the preferred site of ligation was identified (5-6 mm proximal to the trifurcation), and partial ligation was performed by tightly tying a suture (8-0) to include a 50% cross-section area of the nerve. Closure was carried out with respect to anatomical layers. Procedures in the sham group were performed by completing all incisions and/or manipulations until ligation.

Biochemical Analyses

Blood samples from the tail vein were collected into EDTA-K2-containing vacutainer tubes at baseline (day 0) and immediately before sacrifice (day 3). Complete blood samples were centrifuged (1,500×g, 10 min) to obtain plasma which was stored at -80°C until analyses were performed. KYNA and QA levels were measured using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits according to manufacturer protocols (CED718Ge and CEK552Ge, respectively; Cloud-Cone, China).

Statistical Analysis

All analyses were subject to a $p \leq 0.05$ threshold for statistical significance and were performed on the SPSS v. 25.0 software (IBM Corp., Armonk, NY, USA). For the visualization of data, the GraphPad Prism v. 9.0.0 software (La Jolla, CA, USA) was used. Variables were summarized with median (minimum-maximum) values in tables and with mean±standard error of mean (SEM) values in graphs. Since group sizes would not allow for parametric testing, non-parametric tests were used to perform within-group (Wilcoxon signed ranks) and between-groups (Kruskal-Wallis) comparisons. Post-hoc pairwise corrections were performed with the Bonferroni correction. Directional relationships between continuous variables were assessed by calculation of the Spearman correlation coefficient.

Results

A total of 28 rats underwent the respective experimental protocols; however, two rats died within the 72-hour period after the procedures. One was in the high-dose diclofenac group and the other was in the normal-dose diclofenac group.

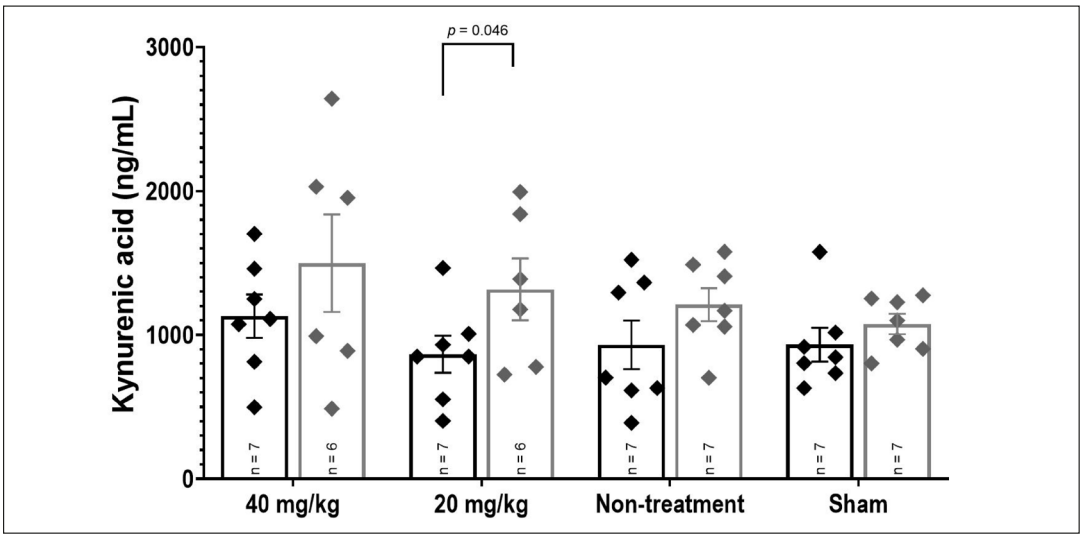

Figure 1. Comparison of kynurenic acid levels between groups and between baseline (black) & third-day (grey) measurements.

Between-groups comparisons revealed that all analyzed parameters were similar at baseline in all groups (p>0.05). Right-side von Frey results showed that third-day values were significantly lower in the high-dose diclofenac group compared to the normal-dose diclofenac group (p=0.018). Other comparisons of third-day values were similar in all groups (p>0.05) (Table I).

Within-group comparisons revealed that normal-dose diclofenac recipients had significantly higher KYNA concentration (p=0.046) and higher KYNA-to-QA ratio (p=0.028) on the third day compared to baseline (Figure 1). Baseline and third-day QA levels were similar in all groups (p>0.05). Hot plate response demonstrated a significant change in only the non-treatment group, with shorter response time on the third day (p=0.027) (Figure 2). The high-dose diclofenac group had significantly lower day 3 von Frey thresholds bilaterally (p=0.042 for both); whereas, in the normal-dose diclofenac group, post-intervention right-sided values were significantly higher (p=0.041) but left-sided values were similar (p=0.336), when compared to baseline results

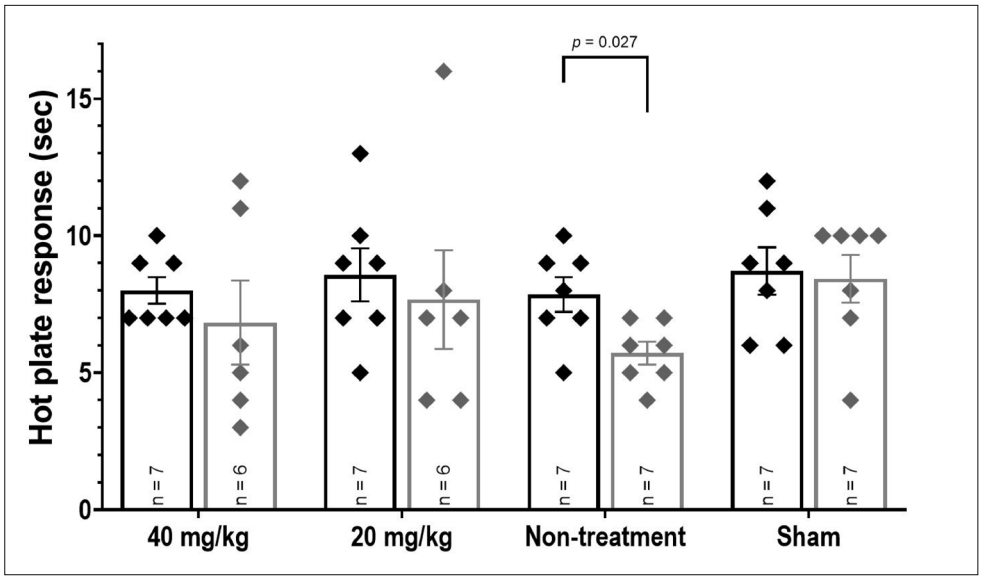

Figure 2. Hyperalgesia results, comparisons between groups and between baseline (black) & third-day (grey) measurements.

Table I. Within-group and between-groups comparison results.

Parameters	High-dose diclofenac (40 mg/kg)	Normal-dose diclofenac (20 mg/kg)	Non-treatment	Sham	*p*-value
KYNA, day 0 (ng/mL)	1,111.2 (498.8-1,702.2)	850.6 (403.4-1,465.9)	703.4 (389.8-1,521.8)	845 (630.4-1,577.1)	0.629
KYNA, day 3 (ng/mL)	1,472.9 (487.9-2,642.3)	1,659.3 (724.7-1,993.4)	1,168 (702.2-1,578)	1,100.5 (803.1-1,275.5)	0.851
p-value (paired)	0.249	**0.046**	0.128	0.237	
QA, day 0 (ng/mL)	8.12 (6.98-13.59)	9.04 (6.83-10.31)	8.76 (6.43-10.42)	8.6 (7.65-13.54)	0.853
QA, day 3 (ng/mL)	9.92 (8.5-13.04)	7.86 (5.53-14.19)	10.84 (5.69-12.36)	9.93 (6.25-15.43)	0.371
p-value (paired)	0.116	0.753	0.063	0.091	
KYNA-to-QA ratio, day 0	132.4 (57.3-208.4)	95.6 (53.7-158.5)	86.95 (60.6-164.5)	87.19 (62.4-192.1)	0.616
KYNA-to-QA ratio, day 3	152.3 (37.4-278.1)	181.5 (54.9-250.1)	129.88 (56.8-188)	110.05 (52.1-196.5)	0.523
p-value (paired)	0.463	**0.028**	0.499	0.499	
Hot plate response, day 0 (sec)	7 (7-10)	9 (5-13)	8 (5-10)	9 (6-12)	0.916
Hot plate response, day 3 (sec)	5.5 (3-12)	7 (4-16)	6 (4-7)	10 (4-10)	0.314
p-value (paired)	0.400	0.586	**0.027**	0.932	
Right-side von Frey, day 0 (F)	60 (60-180)	60 (26-100)	60 (26-100)	60 (26-100)	0.394
Right-side von Frey, day 3 (F)	43 (26-100)[a]	100 (60-180)[b]	60 (60-100)[a,b]	60 (60-100)[a,b]	**0.018**
p-value (paired)	**0.042**	**0.041**	1.000	1.000	
Left-side von Frey, day 0 (F)	100 (60-180)	60 (26-60)	60 (26-100)	60 (26-100)	0.065
Left-side von Frey, day 3 (F)	43 (10-100)	60 (26-100)	26 (10-60)	60 (10-180)	0.372
p-value (paired)	**0.042**	0.336	**0.042**	0.731	

KYNA: kynurenic acid, QA: quinolinic acid, F: force in grams. Data are described with median (minimum-maximum) values, *p*-values in bold indicate statistical significance. Paired tests were performed with the Wilcoxon Signed Ranks test, independent groups were compared with the Kruskal-Wallis test. Post-hoc corrections were performed with the Bonferroni method; same-letter notations were used to show the lack of statistical significance in pairwise comparisons between the respective groups.

(Table I and Figure 3). The non-treatment group had significantly lower von Frey thresholds on the third day when compared to baseline (p=0.042) (Figure 3). It must be noted that the sham group did not demonstrate any significant changes from baseline to the third day in any of the parameters (Table I). Finally, we did not identify any notable correlations between the analyzed variables in any of the groups.

Discussion

The nociceptive findings of our study show that the neuropathic pain model was indeed effective in creating neuropathic pain, as demonstrated by the significant decreases (from day 0 to day 3) in hot plate response and left-sided von Frey thresholds observed in the non-treatment group. Although KYNA levels were relatively increased after surgery in all groups, diclofenac treatment was found to be associated with a significant increase in KYNA level and KYNA-to-QA ratio from day 0 to day 3 when administered at 20 mg/kg/day. Perhaps more importantly, in contrast to the non-treatment group, normal-dose diclofenac recipients had similar left-sided von Frey thresholds on days 0 and 3, suggesting a beneficial effect of treatment. Considering the changes in KYNA levels in normal-dose diclofenac recipients, it may be plausible to infer that the alterations in nociceptive findings were associated with the influence of diclofenac on the kynurenine pathway. However, these positive effects were not observed in the high-dose diclofenac group, showing that either diclofenac was causing adverse effects at this concentration or that the observed effect was not dose-dependent.

Several prior studies[23-27] have investigated diclofenac treatment in neuropathic pain. While early studies[23] evaluating single-dose diclofenac appear to show no efficacy, long-term or daily administration demonstrates positive results in terms of nociceptive findings. One study by Plaza-Villegas et al[24] utilized a model of infraorbital nerve neuropathy and the results showed that systemic pregabalin (300 mg/kg) and systemic diclofenac (1 mg/kg) had beneficial effects on tactile allodynia (von Frey thresholds) measured on days 8, 9 and 10 after neuropathy. However, although pregabalin reduced hyperalgesia, systemic diclofenac was not found to induce this effect[24] – presumably in relation with the low dosage of treatment. In another study[25], an intraperitoneal diclofenac administration of 2 mg/kg/day for 10 days was found to significantly improve paw withdrawal threshold in a rat model of tibia injury, and there appeared to be a dose-dependent increase in measured thresholds from lower to higher dosage (0.25, 0.5, and 1 mg/kg/day). Of note, the authors of this study suggested a potentiating effect with the combination of diclofenac and vitamin B12[25]. This relationship between B12 and diclofenac has also been examined by Granados-Soto et al[23], who found an

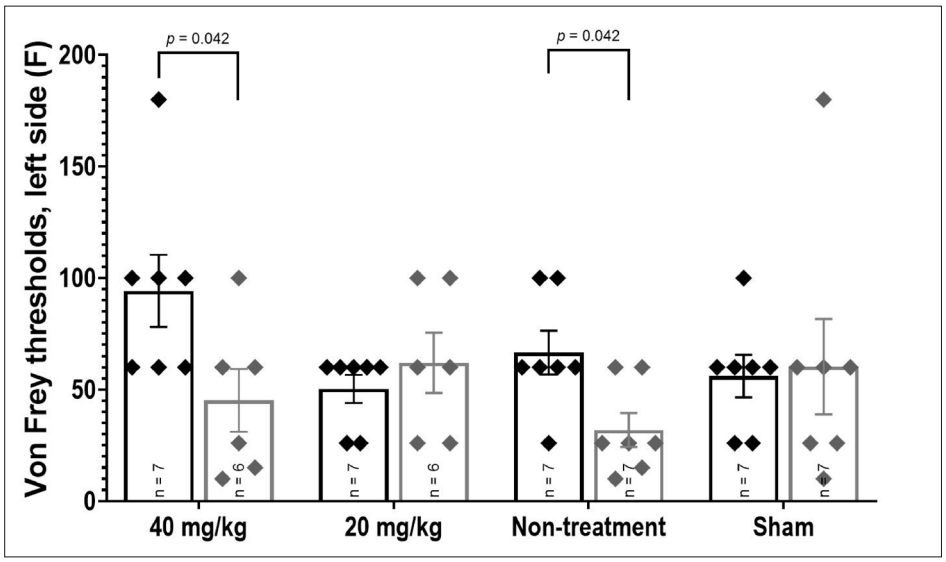

Figure 3. Allodynia results, comparisons between groups and between baseline (black) & third-day (grey) measurements.

anti-allodynic effect for B12 but did not report a beneficial effect of diclofenac in neuropathic pain (1-10 mg/kg) – let alone a potentiating relationship between the compounds[23]. The neuropathic pain model in the mentioned study was created by spinal ligation (L5-L6); therefore, the peripheral mechanism of diclofenac may not have been observed, circulatory levels may not have changed, or dosage may have been too low to confer central effects[23]. After it became clear that diclofenac was not only an analgesic that induced peripheral effects but had considerable effects on the central nervous system[17,18], researchers sought to compare diclofenac with other centrally-effective drugs in models of neuropathic pain. For instance, the study by Ibrahim et al[26] evaluated cold allodynia and hot plate tests in a rat model of neuropathic pain induced *via* sciatic nerve ligation, which is a very similar design to ours. The authors reported that 10 mg/kg/day diclofenac alone exerted similar anti-allodynic and anti-hyperalgesic effects compared to two other agents, 10 mg/kg/day celecoxib (selective COX-2 inhibitor) and 100 mg/kg/day gabapentin[26]. They also found that the combination of these NSAIDs with gabapentin induced greater improvements relative to the respective monotherapies[26]. In the present study, we found that allodynia was alleviated in the normal-dose diclofenac group, demonstrating its protective effect against neuropathic pain. Also, we found that normal-dose diclofenac therapy resulted in higher levels of KYNA and KYNA-to-QA ratio, suggesting a potential relationship between the metabolites of the kynurenine pathway and diclofenac therapy. However, high-dose diclofenac (40 mg/kg/day) did not induce similar effects. This latter result is somewhat in contrast with the findings reported by Edwards and Mather[27] who showed that 40 mg/kg dosage of diclofenac (subcutaneous) caused a significantly greater accumulation of kynurenine and KYNA in tissues when rats were treated with tryptophan prior to diclofenac administration.

It is crucial to note that the current study was based on the hypothesis that diclofenac could be exerting protective effects through its influence on the kynurenine pathway by altering the levels of KYNA and QA, which are metabolites that have opposing effects on NMDA receptor activity[11]. The literature concerning the pain-related effects of kynurenine pathway metabolites describes interesting relationships. KYNA levels have been shown to be reduced in migraine[28,29], while QA elevation has been identified as a primary biomarker in patients with chronic pain[30]. Also, neuropathic pain itself is suggested to cause a physiological change favoring KYNA production relative to QA production[11], which is somewhat supported by our results showing relatively increased KYNA concentrations in all groups. Nonetheless, previous studies[26,31,32] have suggested other potential mechanisms to explain the effects of NSAIDs in neuropathic pain, including regulation of protein kinase B signaling[31], modulation of prostaglandin receptors[32], and changes in the levels of prostaglandins and interleukins within the immediate environment of injury[26]. These mechanisms may have contributed to the anti-allodynic and anti-hyperalgesic effects of diclofenac that were demonstrated in this study. Interestingly, rats with high-dosage treatment did not demonstrate a significant increase in KYNA levels in our study. This may have been because of tryptophan depletion due to the fact that we did not administer tryptophan pretreatment or tryptophan-enriched diet. However, it is also possible that an exceedingly high dosage of diclofenac may have triggered oxidative stress and toxicity[33,34], explaining the lack of protective effects with this dosage.

Limitations

As mentioned above, a primary limitation of this study is the fact that we cannot demonstrate a direct link between the nociceptive results of diclofenac therapy and the changes in KYNA or QA levels due to the lack of dose-dependent effects and absence of analyses concerning other possible pathways and/or mediators. Nonetheless, the present study demonstrated that KYNA level and KYNA-to-QA ratio were increased among recipients of 20 mg/kg/day diclofenac treatment. Taken together with the von Frey results (non-treatment and normal-dose diclofenac groups), it appears that diclofenac therapy may, at least in part, exert its anti-allodynic and anti-hyperalgesic effects by altering the levels of metabolites in the kynurenine pathway. Another limitation is the fact that we only measured circulatory levels of KYNA and QA, which may not accurately reflect central concentrations. Future studies would benefit from expanding upon the current research by measuring kynurenine metabolites in relevant tissues, by quantifying the activities of enzymes involved in the kynurenine pathway, by performing longer studies with intermittent measurements, and possibly, by providing rats with tryptophan-enriched diet

or tryptophan pretreatment. Also, exploring other possible mechanisms of diclofenac in the regulation of neuropathic pain may be valuable.

Conclusions

The current study revealed that, compared to non-treatment, 20 mg/kg/day diclofenac therapy improved nociceptive findings in a rat model of neuropathic pain created by partial ligation of the sciatic nerve. We also found that this treatment resulted in significantly elevated levels of KYNA and significantly higher KYNA-to-QA ratio; however, nociceptive results were not correlated with the levels of KYNA or QA, and these beneficial effects conferred by diclofenac did not appear to be dose-dependent.

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Availability of Data and Materials
The datasets generated during and/or analyzed during the current study are not publicly available due to ongoing applications for funding but are available from the corresponding author on reasonable request.

Conflict of Interests
The authors declare that they have no conflict of interests.

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None.

Ethics Approval
The experimental protocol of this study was approved by the Kobay Animal Studies Laboratory Ethics Committee (approval code: 541, date: April 20, 2021).

Informed Consent
Not applicable.

Authors' Contributions
S. Debbag: planning, design, data collection, literature survey, interpretation of the results, preparation of manuscript draft, and active intellectual support. A. Yalcinkaya: design, data collection, biochemical and statistical analyses, and preparation of manuscript draft. F. Saricaoglu: design, data interpretation, and writing contribution. All authors have made critical revisions to the article, contributed to the intellectual content, and approved the final version of the article to be published.

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