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# The Effect of L-NAME and Celecoxib on Diabetic Neuropathy in Rats

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**Abstract**

**Background:** Diabetic neuropathy (DN) is known as the most troublesome of diabetes mellitus complications. There is a cross-talk between cyclooxygenase-2 (COX-2) and the enzyme NO synthase (NOS) in pain pathophysiology in the dorsal root ganglia and the spinal cord. This study aimed to determine the possible role of the NOS inhibitor, N(ω)-nitro-L-arginine methyl ester (L-NAME), or the COX-2 inhibitor, celecoxib, alone and in interaction with each other, on hyperalgesia and allodynia in rats with DN.

**Methods**: Streptozotocin (STZ) (60 mg/kg, IP, once) was used to induce diabetes in male Wistar rats. After 72 hours, the animals were divided into groups that received celecoxib (5 mg/kg), L-arginine (L-ARG) (50 mg/kg), or L-NAME (50 mg/kg) alone and two groups that received a combination of celecoxib with either L-ARG or L-NAME. The von Frey and acetone tests were used to evaluate hyperalgesia and allodynia 14 days after treatment.

**Results**: A significant increase in the withdrawal threshold level was observed in the groups receiving celecoxib alone ($P<0.001$) and in combination with L-ARG ($P<0.001$) or L-NAME ($P<0.001$). The reaction percentage in the acetone test significantly decreased in the celecoxib, L-NAME, celecoxib + L-ARG, and celecoxib + L-NAME groups ($P<0.001$) compared with the diabetic control group.

**Conclusion**: The main finding was that inhibiting COX-2 and NOS reduced hyperalgesia and mechanical allodynia in diabetic rats. Also, the results revealed that there is cross-talk between these two enzymes.

**Keywords:** Celecoxib, Nitric oxide, Cyclooxygenase-2, Diabetes mellitus, Neuropathy, L-NAME

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## Introduction

Diabetes mellitus is a global public health issue (1), and knowing the complications related to diabetes is becoming more important (2,3). Diabetic neuropathy (DN) is known as the most common and troublesome of these complications. DN leads to increased mortality and substantial morbidity due to infection, pain, limitation of daily activities, and psychosocial consequences, and consequently, it imposes a heavy economic burden on diabetic patients (4). Neuropathic pain is "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (5,6). Hypersensitivity and imbalance between excitatory and inhibitory signals within the spinal cord postulate this problem (7). Allodynia and hyperalgesia are two kinds of hypersensitivity to painful stimuli (8,9). Cyclooxygenase (COX) is a homodimer with a molecular mass of 71 kDa and prostaglandin synthase and hydroperoxidase activities (10). Several studies have demonstrated that COX isoenzymes could induce the production of prostaglandin, one of the primary mediators in inflammation and pain (11,12).

The anti-inflammatory activity of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) like celecoxib is due to the inhibition of COX (13). Both types of COX, i.e., cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), are inhibited by most NSAIDs; however, celecoxib selectively inhibits COX-2 (14).

Nitric oxide (NO) is a highly reactive molecule in mammalian cells. The enzyme NO synthase (NOS) plays the leading role in its production (15). Both NO and prostaglandin pathways play roles in inflammatory conditions. Previous studies have claimed that NO can activate COX isoforms, and NSAIDs such as aspirin and indomethacin can significantly reduce NOS activity (16,17). On the other hand, it has been shown that the nitric oxide produced by the activity of iNOS increases the effect of indomethacin (18). Therefore, it is evident that there is a cross-talk between COX and NO. Despite evidence in the mutual and cyclic relationships of enzymes involved in pain induction, there are not enough findings about the interaction between these two types of mediators in DN-level convergence. This study aimed to evaluate the possible

role of the NOS inhibitor, L-NAME, and the interaction between celecoxib and L-NAME on antihyperalgesic and antiallodynic neuropathy in diabetic rats.

## Methods

### *Animals and grouping*

All trials were executed following the *Guidelines for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1985), and the Research and Ethics Committee of Kashan University of Medical Sciences has approved this study (Ethical code: No 29/5/1/4865). Eighty male Wistar rats (200 ± 20 g) 6–8 weeks old were kept in the animal house of the Physiology Research Center, Kashan University of Medical Sciences. The animals were kept in standard polypropylene cages at standard temperature (25 ºC), 50%–60% humidity, and a 12/12 hours light/dark cycle. The animals had free access to standard feed and water. They were divided randomly into eight groups ($n = 10$ in each group). Two non-diabetic groups: control (intact), with no intervention (CTL), and sham, which received only 1 mg/kg citrate buffer intraperitoneally as streptozotocin (STZ) solvent on the day of diabetes induction (19,20). The six diabetic groups included the diabetic control group, which received STZ 60 mg/kg once, intraperitoneally (IP), the Celecoxib group, which received celecoxib 5 mg/kg by gavage (14 days) (21) from the first day after STZ injection, the L-arginine group, which received L-arginine 50 mg/kg, IP, for 14 days, starting from the day after STZ injection, the L-NAME group, which received L-NAME 50 mg/kg, IP, for 14 days, starting from the day after STZ injection, the celecoxib + L-arginine group, which received celecoxib (5 mg/kg, gavage) 60 minutes before treatment with L-arginine (50 mg/kg, IP) for 14 days after diabetes induction, and the celecoxib + L-NAME group, which received celecoxib (5 mg/kg, gavage) 60 minutes before treatment with L-NAME (50 mg/kg, IP) for 14 days after diabetes induction (19,20). In all groups, the day of STZ injection was considered day zero, and the number of treatment days was calculated from the following day.

### *Drugs*

The following drugs were used: STZ was purchased from Enzo Life Sciences Co. (Germany), L-arginine and L-NAME from Sigma-Aldrich Co. (USA), and celecoxib from DarouPakhsh Co. (I.R. Iran). Celecoxib was suspended in carboxymethyl cellulose (0.5%, w/v) (21) and delivered by gavage once daily. L-arginine and L-NAME were dissolved in normal saline and injected IP.

### *Induction and assessment of diabetes in rats*

A single IP injection of STZ was used for the induction of diabetes. The STZ solution was freshly prepared by dissolving STZ in citrate buffer, and 60 mg/kg was injected IP (22). Diabetes induction was assessed by measurement of the tail vein blood glucose level in three steps: placing the animal in the restrainer, finding the tail vein and making a slit using a scalper, taking a drop of blood and measuring the blood glucose with glucometer (HEA-214, OMRON, USA) strips 72 hours after STZ injection. Only rats with blood glucose concentrations exceeding 220 mg/dL (19) were considered diabetic.

### *Behavioral testing (mechanical and thermal thresholds)*

All behavioral tests started 72 hours after the STZ injection and continued for 14 days. In order to evaluate the level of pain sensitivity in rodents, two practical tests, the acetone test and the von Frey test, were used (23,24).

### *Von Frey test*

The threshold of lifting the paw against the sensation of mechanical stimuli produced by von Frey filaments is considered a criterion for evaluating mechanical sensitivity (Stolting, Wood Dale, IL, USA) (25). Briefly, the animals were in separate plastic chambers with dimensions of 18 × 18 × 25 cm and moved freely on a wired screen with 6 $mm^2$ apertures; they were allowed to adapt for 15 minutes before performing the test. Von Frey filaments calibrated (Stolting Inc., Wood Dale, and IL) with forces of 2 to 60 grams were randomly used to create mechanical stimuli to the backs of the animals' right and left paws. Each filament was used ten times, and the number of times the claws were withdrawn was recorded (24).

### *Acetone test*

The evaporative cooling acetone test measured cold sensitivity by spraying a small amount of of acetone with a syringe on the plantar surface of the hind paws, with only the drop of acetone in contact with the paw and not the tip of the syringe. The duration of paw withdrawal was recorded for 30 seconds after that. This process was repeated at each time point (30 minutes, 1 , 2, 4, and 6 hours post-injection), and the repetition of foot withdrawal was recorded as a percentage. A drop of water at a temperature of 37 °C was used as a control (26).

### *Statistical analysis*

All data were reported as mean ± standard error of the mean (SEM), and differences were considered significant if the *P*-value was less than 0.05. One-way ANOVA was used to compare the quantitative behavioral response data, followed by Tukey's HSD post hoc test.

## Results

Higher fasting blood glucose level was observed in diabetic groups (493 ± 10 mg/dL) compared to control (170.14 ± 11 mg/dL) ($F_{2,18} = 26.476$, $P < 0.001$) (Figure 1). Measuring the pain behavior revealed that compared to control group, the diabetic control group showed a smaller threshold value in the von Frey test ($F_{2,60} = 52.048$, $P < 0.001$)

(Table 1). Furthermore, one-way ANOVA revealed an increase in the percentage of reaction in the acetone test ($F_{2,60}=65.495$, $P<0.001$) in comparison with the control groups (Table 1).

***Effect of chronic administration of celecoxib and L-NAME on response to the von Frey test in diabetic rats***

In the von Frey test, one-way ANOVA showed a significant difference among the treated groups [F(3,24) = 25.47, $P<0.001$] (Figure 2). Post-hoc analysis revealed that rats treated by celecoxib, L-NAME, and also celecoxib + L-NAME showed a significantly higher withdrawal threshold than the diabetic control group ($P<0.001$). In addition, the analyzed data indicated a significant difference between the group that received celecoxib alone and rats treated with both celecoxib and L-NAME simultaneously ($P<0.001$). This data revealed that NOS and COX-2 inhibition attenuated allodynia in rats with diabetes.

***Effect of chronic administration of celecoxib and L-NAME on acetone-evoked behavior in diabetic rats***

The acetone test data analysis showed that there was a significant difference between the treated groups [F(3,24) = 31.32, $P<0.001$) (Figure 3). Post hoc analysis revealed that celecoxib (22.85 ± 22.85), L-NAME (18.57.1.5), and celecoxib + L-NAME (11.42 ± 1.4) could decrease withdrawal reaction in the acetone test compared to diabetic controls (74.28 ± 3.68). There was a significant difference between the group that received celecoxib alone and rats treated with both celecoxib and L-NAME simultaneously ($P<0.05$). This data revealed that both celecoxib and L-NAME attenuated allodynia in rats with diabetes.

**Table 1.** The effect of STZ on threshold value in the von Frey test and percentage of reaction in the acetone test

| Group | The threshold value in the von Frey test | Percent of reaction in the acetone test |
|---|---|---|
| Control | 60 ± 0.1 | 18 ± .05 |
| Diabetic | 7 ± 0.08*** | 79 ± 0.2*** |

The response was determined 14 days after STZ injection. The results of one-way ANOVA analysis are reported as mean ± SEM ($n$ = 8–10). ***$P<0.001$ versus CTL.

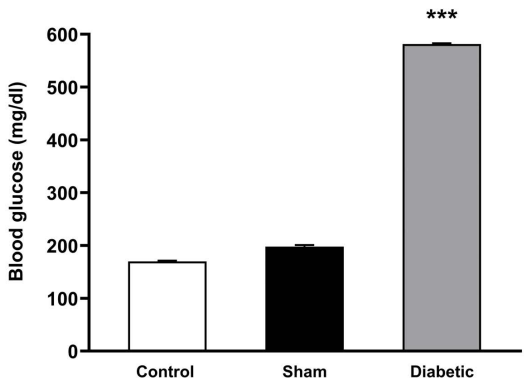


**Figure 1.** The effect of STZ injection on blood glucose levels. The blood glucose concentration was identified on the third day after the STZ injection. The results of one-way ANOVA analysis are reported as mean ± SEM ($n$ = 8–10). ***$P<0.001$ versus CTL

***Effect of chronic administration of celecoxib and L-arginine on response to the von Frey test in diabetic rats***

In the von Frey test, one-way ANOVA revealed a significant difference among the treated groups [F(3,24) = 175.744, $P<0.001$] (Figure 4). Post-hoc analysis revealed that rats treated with celecoxib (24.42 ± 1.57) and celecoxib + L-arginine (26 ± 0.1) showed a significant difference in withdrawal threshold compared to the diabetic control group ($P<0.001$). In addition, the analyzed data revealed no significant difference among groups that received L-arginine alone and diabetic control rats.

***The effect of chronic administration of celecoxib and L-arginine on acetone-evoked behavior in diabetic rats***

In the acetone test, data analysis showed that there was a significant difference between the treated

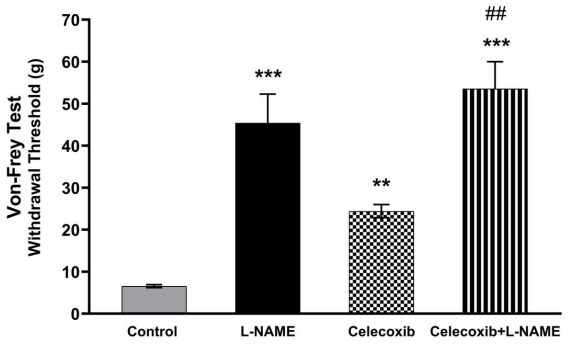


**Figure 2.** The effect of L-NAME (50 mg.kg$^{-1}$, IP) and celecoxib (5 mg.kg$^{-1}$, IP) alone and administered together on withdrawal threshold in the von Frey test in STZ-induced diabetic rats. The response was evaluated 14 days post-STZ injection. The results of one-way ANOVA analysis are reported as mean ± SEM ($n$ = 8–10). ***$P<0.001$ and **$P<0.01$ versus CTL. ## $P<0.01$ compared with the celecoxib group

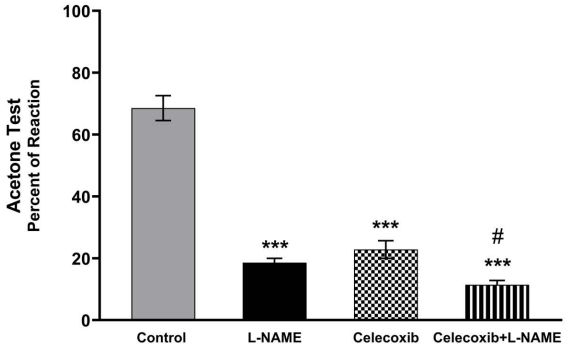


**Figure 3.** The effect of L-NAME (50 mg.kg$^{-1}$, IP), celecoxib (5 mg.kg$^{-1}$, IP), and their combined administration on the reaction percentage in the acetone test in STZ-induced diabetic rats. The response was evaluated for 14 days post-STZ injection. The results of one-way ANOVA analysis are expressed as mean ± SEM ($n$ = 8–10). ***$P<0.001$ versus the control group. # $P<0.05$ compared with the celecoxib group

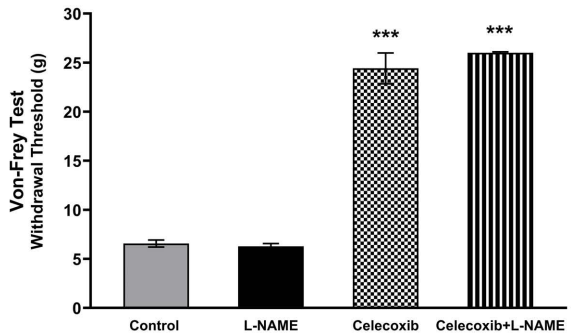


**Figure 4.** The effect of L-ARG (50 mg.kg$^{-1}$, IP), celecoxib (5 mg.kg$^{-1}$, IP), and their combination on withdrawal threshold in the von Frey test in STZ-induced diabetic rats. The response was evaluated for 14 days post-STZ injection. The results of one-way ANOVA analysis are expressed as mean ± SEM ($n$ = 8–10). ***$P$ < 0.001 compared with CTL

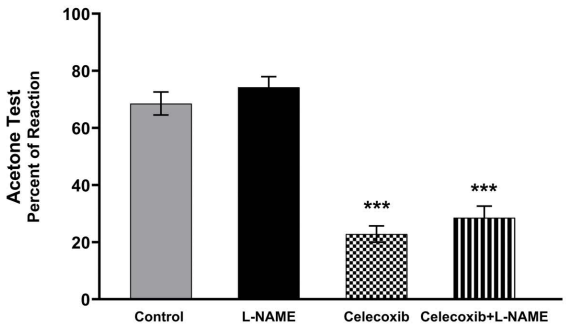


**Figure 5.** The effect of L-ARG (50 mg.kg$^{-1}$, IP), celecoxib (5 mg.kg$^{-1}$, IP) and their interaction on the reaction percentage in the acetone test in STZ-induced diabetic rats. The response was evaluated for 14 days post-STZ injection. The results of one-way ANOVA analysis are expressed as mean ± SEM ($n$ = 8–10). ***$P$ < 0.001 versus CTL

groups [F(3,24) = 52, $P$ < 0.001) (Figure 5). Post-hoc analysis revealed that celecoxib (22.85 ± 22.85) and celecoxib + L-ARG (28.57 ± 4.4) decreased withdrawal reactivity in the acetone spray compared to the diabetic control group (74.28 ± 3.68). Meanwhile, no significant difference was observed between groups that received L-arginine alone and diabetic control rats.

## Discussion

The main finding of this study was that inhibition of the COX-2 and NOS enzymes reduced mechanical allodynia and hyperalgesia in rats with diabetes. In addition, simultaneous inhibition of both enzymes potentiated this effect. Also, our data confirm the interaction of COX-2 and NOS because inhibition of COX-2 potentiated the effect of L-arginine on hyperalgesia and allodynia in diabetic rats.

Our findings show that the activity of NO is complicated in the pathogenesis of diabetic neuropathic pain. Previous studies have shown that NO has a stimulatory effect on the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6, and nuclear factor-κB (NF-κB) (27). Also, based on other research, neuropathic pain is caused by increased levels of all these inflammatory mediators (28-30).

The fact that both acute and chronic inhibition of the NOS enzyme reduces pain-induced responses in animal pain models can be inferred from the results of our study and many other studies (31). In one study, L-NAME (which inhibits all three isoforms of NOS) induced significant antinociceptive events on nociceptive thresholds in acute pain and altered mechanical allodynia and thermal hyperalgesia in rats subjected to chronic contraction injury (32). Several studies have examined the involvement of NO in acute and chronic pain models in mice that lack different isoforms of NOS genes. Kuboyama and colleagues' study showed that in the absence of different types of NOS, spinal microglia and physical allodynia are not activated after nerve tissue damage (33).

The findings of other studies suggest that neuronal NOS (nNOS) is required to maintain normal peripheral nerve function and small sensory nerve fiber innervations (34,35). Depending on the local NO concentration, this factor may have beneficial or harmful effects. In physiological concentrations, the protective effects of NO are mediated by the activation of cyclic GMP (36). On the other hand, the overproduction of NO, induced by isoforms of NOS, leads to pathological procedures such as neurotoxicity, neuropathic pain, and septic shock. Overactivation of the N-methyl-D-aspartate (NMDA) receptor and peroxynitrite formation results in high levels of nNOS and endothelial NOS (eNOS), suggesting that nNOS (34) and eNOS (37) are critical to pain hypersensitivity.

Pain perception depends on the amount of the painful agent at the site of inflammation, handling and conduction in the spine, and processing in the upper spinal nerve sections. In this study, systemic administration of the L-NAME resulted in NOS inhibition and decreased NO levels in the inflammation or spinal cord site.

In addition, this study showed that celecoxib could decrease mechanical and chemical allodynia. Previous studies have claimed that COX-2 can regulate neuropathic pain in the spinal cord and dorsal root ganglion (38). On the other hand, two dedicated COX-2 inhibitors, celecoxib (39) and meloxicam, reduced allodynia and hyperalgesia in rats (40). Inhibition of PI3K/Akt2 in dorsal root ganglia and ERK1/2 pathways in the spinal cord by celecoxib reduces oxaliplatin-induced neuropathic pain in mice (41). Our findings have shown the specific role of celecoxib in reducing hyperalgesia and physical allodynia caused by diabetes. In addition, combining celecoxib and L-NAME strengthened these analgesic effects in diabetic rats. Nowadays, it is clear that in the NOS and COX pathways, there is a "cross-talk" among the products (42). Also, Needleman's report showed that NO can activate

COX (43). *In vitro* studies show that NO, exogenous or donor, can encourage COX-1 and COX-2 activity (44,45). A significant decrease in NOS activity has been observed under the influence of aspirin and indomethacin from the class of NSAID drugs; this indicates a two-way interaction between NO and prostaglandin pathways (16).

## Conclusion

In conclusion, our data confirm and extend previous results that administration of L-NAME or celecoxib alone can attenuate neuropathic pain.

This interaction can be considered in the revision of treatment protocols to reduce symptoms caused by diabetes.

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The data reported in this paper were obtained from a study conducted as a master's thesis project at Kashan University of Medical Sciences.

**Authors' Contribution**

**Conceptualization:** Gholamali Hamidi.
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**Methodology:** Gholamali Hamidi, Azhdar Heydari, Hamidreza Banafshe.
**Project administration**: Gholamali Hamidi, Hamidreza Banafshe, Azhdar Heydari.
**Resources:** Maryam Hosseini, Samaneh Sadat Alavi, Mahsa Hadizade.
**Software:** Mahsa Hadizade, Samaneh Sadat Alavi.
**Supervision:** Gholamali Hamidi, Azhdar Heydari.
**Validation:** Azhdar Heydari, Gholamali Hamidi
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**Competing Interests**

The authors declare that they have no competing interests.

**Ethical Approval**

All trials were executed following the guidelines for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985) and the research and ethics committee of Kashan university of medical Sciences has approved this study (Ethical code: No. 29/5/1/4865).

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