Submitted: 28 April, 2024 Accepted: 09 July, 2024 Published: 08 September, 2024 DOI:10.22514/sv.2024.113

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ORIGINAL RESEARCH

Lorazepam attenuates neuroinflammation by suppressing T cell infiltration in diabetic peripheral neuropathy

Ying Wang[1], Yaping Sui[2], Chunli Xing[1,*]

[1]Department of Geratology, People's Hospital of Lixia District of Jinan, 250014 Jinan, Shandong, China
[2]Medical Record Management Division, Yantai Affiliated Hospital of Binzhou Medical University, 264100 Yantai, Shandong, China

***Correspondence**
chunli_dr@163.com
(Chunli Xing)

Abstract

Diabetic peripheral neuropathy (DPN) is a chronic microvascular complication of diabetes mellitus that results in significant pain and severely affects patients' quality of life. In this context, lorazepam has demonstrated promising pharmacological effects, specifically through its antidepressant and antiepileptic properties, in the management of various diseases. However, the regulatory effects and pathways associated with lorazepam in the progression of DPN remain unclear. In this study, a DPN rat model was successfully established, and further investigations showed a reduction in the exit threshold (g) in DPN rats, which was reversed after lorazepam treatment, indicating that lorazepam can alleviate mechanical allodynia in DPN rats. Additionally, lorazepam treatment reduced inflammation in DPN rats. Furthermore, lorazepam inhibited T cell infiltration in the dorsal root ganglion (DRG) of DPN rats, and the activated nuclear factor kappa-B (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways in DPN rats were suppressed by lorazepam treatment. In conclusion, lorazepam reduced neuroinflammation by inhibiting T cell infiltration, thereby ameliorating DPN. These findings may provide novel insights into the therapeutic potential of lorazepam for the treatment of DPN.

Keywords

Lorazepam; Neuroinflammation; T cell infiltration; Diabetic peripheral neuropathy

1. Introduction

Diabetes mellitus has a high global prevalence and is one of the most common chronic diseases [1]. It can lead to diabetic neuropathy, including diabetic peripheral neuropathy (DPN) [2], which has recently shown increased incidence among the chronic complications of diabetes mellitus [3]. DPN is challenging to manage and imposes substantial burdens on patients and their families. Its pathogenesis is complex, involving gene expression modulation, inflammation and injury repair processes [4]. Research indicates that T cells play a regulatory role in peripheral nerve injury, with increased T cell infiltration exacerbating neuroinflammation [5]. Therefore, identifying effective drugs to reduce T cell infiltration and inflammation levels could significantly benefit DPN treatment.

Lorazepam, a benzodiazepine drug, has demonstrated pharmacological effects such as antidepressant and antiepileptic properties [6, 7]. Despite its potential long-term side effects, lorazepam is widely used for its effective and rapid onset in treating depression [8]. Additionally, lorazepam has been found to play a role in regulating various conditions. For example, it can alleviate burn pain [9] and modulate paired-pulse inhibition of median nerve-stimulated somatosensory evoked potentials [10]. In normal subjects, lorazepam reduces cardiac vagal modulation [11]. However, in pancreatic cancer, lorazepam induces interleukin-6 (IL-6) production and is associated with unfavorable survival outcomes [12]. Importantly, benzodiazepines, including lorazepam, can inhibit lymphocyte effector functions and impair both adaptive and innate immune systems [13, 14]. Despite these known effects, the regulatory impacts and associated pathways of lorazepam in the progression of DPN remain poorly understood.

In this study, it was revealed for the first time that lorazepam attenuates neuroinflammation by inhibiting T cell infiltration, thereby improving DPN through modulation of the NF-κB and Nrf2 pathways. These findings suggest that lorazepam could be an effective therapeutic option for treating DPN.

2. Materials and methods

2.1 Rat model

Sprague-Dawley (SD) rats (n = 18) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

The SD rats were maintained on a high-calorie/sugar diet consisting of 0.5% sodium cholate, 2.0% cholesterol, 5.0% yolk powder, 10.0% lard, 10.0% sucrose and 72.5% normal

diet. Rats with a decreased insulin sensitivity index were selected for further treatment. These rats received a single intraperitoneal injection of streptozotocin (STZ, S0130, 35 mg/kg, Sigma, St. Louis, MO, USA). After two weeks, blood glucose levels stabilized ($\geq$11.1 mmol/L), confirming the establishment of the type 2 diabetes mellitus (T2DM) rat model. Concurrently, significant differences in mechanical and heat pain responses indicated the successful establishment of the DPN rat model.

The rats were divided into three groups: Control group (n = 6, no STZ treatment), DPN group (n = 6), and DPN + lorazepam group (n = 6, DPN rats treated with 0.25 mg/kg lorazepam via intraperitoneal injection).

2.2 Von Frey test

In a plexiglass chamber, the hind paws of rats were stimulated using the up-down method with a Von Frey filament-based pain meter (NC12775, Yuyan Instruments, Shanghai, China). Positive responses included raising of legs, licking of feet or dodging. The withdrawal threshold was defined as the minimum force in grams eliciting a positive response. Post-modeling, withdrawal thresholds (g) were assessed on days 0, 3, 7 and 14.

2.3 Enzyme-linked immunosorbent assay (ELISA)

Serum levels of interleukin-6 (IL-6, ab234570, Abcam, Shanghai, China), tumor necrosis factor-α (TNF-α, ab46070) and IL-1β (ab100768) were measured using respective ELISA kits.

2.4 Immunofluorescence (IF) assay

Spinal cord sections (L4–6) were embedded in paraffin and sliced into 4 μm sections. Briefly, the sections were blocked with 10% goat serum containing TritonX-100 (0.3%) and incubated with primary antibody against Cluster of Differentiation 3 (CD3, 1/10, ab135372, Abcam, Shanghai, China) at 4 °C for 12 hours, which was followed by incubation with secondary antibody (1/1000, ab150080) for 2 hours. 4,6-diamino-2-phenyl indole (DAPI) solution was used for nuclear staining, and fluorescent images were captured using a fluorescence microscope (Ni-U/Ni-E, Nikon, Tokyo, Japan).

2.5 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from (DRG) was isolated using a Trizol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from RNA using the PrimeScript™ RT Reagent Kit (RR037A, Takara, Dalian, Liaoning, China). Quantitative real-time PCR (qPCR) was performed using the SYBR Green PCR kit (QPK-201, Toyobo, Osaka, Japan), and mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

The primers were shown:

CD3:

F, 5′-ATGCGGTGGAACACTTTCTGG-3′,

R, 5′-GCACGTCAACTCTACACTGGT-3′;

CD4:

F, 5′-AGGTGATGGGACCTACCTCTC-3′,

R, 5′-GGGGCCACCACTTGAACTAC-3′;

CD8:

F, 5′-CCGTTGACCCGCTTTCTGT-3′,

R, 5′-CGGCGTCCATTTTCTTTGGAA-3′;

C-X-C motif chemokine ligand 10 (*CXCL10*):

F, 5′-CGGTGAGCCAAAGAAGGTCTA-3′,

R, 5′-CTAGCCGCACACTGGGTAAA-3′;

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*):

F, 5′-GCCACAACGACCCCTTCATG-3′,

R, 5′-TGCCAGTGAGCTTCCCGTTC-3′.

2.6 Western blot

On the final day, 6 hours post-administration, rats were euthanized and nervous tissues were collected. Proteins were extracted from nervous tissues using radio immunoprecipitation assay (RIPA) lysis buffer. Subsequently, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to separate proteins, followed by transfer onto polyvinylidene difluoride membranes (PVDF, Sigma, St Louis, Missouri, USA). Primary antibodies against p-p65 (1/1000; ab76302), p65 (0.5 μg/mL; ab16502), Nuclear factor erythroid-2-related factor 2 (Nrf2, 1/500; ab313825), Heme Oxygenase 1 (HO-1, 1/2000; ab189491), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, 1/1000; ab8245) were applied and incubated for 12 hours, followed by incubation with secondary antibody (1/1000; ab6721) for 2 hours. Blots were visualized using an enhanced chemiluminescence kit (89880, Thermo Fisher Scientific, Waltham, MA, USA), and band quantification was performed using Image J software (National Institutes of Health, USA).

2.7 Flow cytometry

Cells from nervous tissues were resuspended and labeled with 10 μL of fluorescein isothiocyanate (FITC)-labeled CD45 antibody (ab210225, Abcam, Shanghai, China) and CD3 antibody (ab34722, Abcam, Shanghai, China). Following a 20-minute incubation at 4 °C in the dark, cells were sorted using FACS Calibur (BD FACSCALIBUR, BD Biosciences, Allschwil, Basel-Landschaft, Switzerland) and analyzed using BD FACS software (CellQuest, BD Biosciences, Allschwil, Basel-Landschaft, Switzerland).

2.8 Statistical analysis

All data are presented as mean $\pm$ standard deviation. Statistical analysis was conducted using GraphPad Prism software, version 9.0 (GraphPad Software, La Jolla, CA, USA). Data were confirmed to follow a normal distribution. Group comparisons were performed using one-way Analysis of Variance (ANOVA), with statistical significance set at $p < 0.05$.

3. Results

3.1 Lorazepam ameliorated mechanical allodynia in DPN rats

Following the successful establishment of the DPN rat model, our investigations showed that the withdrawal threshold (g) was decreased in DPN rats, and this response could be mitigated by lorazepam treatment (Fig. 1). These findings showed that lorazepam can effectively ameliorate mechanical allodynia in DPN rats.

3.2 Lorazepam alleviated inflammation in DPN rats

Next, we investigated the regulatory effects of lorazepam on inflammation in the DPN rats. ELISA results showed that the levels of TNF-α, IL-1β and IL-6 (pro-inflammatory markers) in the serum of DPN rats were elevated, and these effects were counteracted after lorazepam treatment (Fig. 2). Overall, lorazepam alleviated inflammation in DPN rats.

3.3 Lorazepam suppressed T cell infiltration in DPN rats

The regulatory effects of lorazepam on T cell infiltration in DPN were investigated. The results showed that the CD3 level, which was elevated in DPN rats, was significantly reduced following lorazepam treatment (Fig. 3A). Moreover, the mRNA expressions of *CD3*, *CD4*, *CD8* (T cell markers), and *CXCL10* (inflammatory/chemotactic marker) were increased in DPN rats but were normalized after lorazepam treatment (Fig. 3B). Additionally, the increased percentage of $CD45^+$ $CD3^+$ cells in DPN rats was reduced after lorazepam treatment (Fig. 3C). Collectively, these results indicate that lorazepam can effectively suppress T cell infiltration in DPN rats.

3.4 Lorazepam retarded the NF-κB and Nrf2 pathways

Considering that the NF-κB and Nrf2 pathways play essential roles in inflammation, the effects of lorazepam on these pathways were examined. The results revealed that the protein expressions of p-p65/p65 (NF-κB subunit), Nrf2 (a marker of oxidative stress in neurons), and HO-1 (neuroprotective against oxidative stress) were elevated in DPN rats. However, these elevations were reversed following lorazepam treatment (Fig. 4). These data indicate that lorazepam inhibits the activation of the NF-κB and Nrf2 pathways.

4. Discussion

Lorazepam has demonstrated promising pharmacological effects in various diseases [9–12]. However, its regulatory impacts and associated pathways in the progression of DPN remained unclear. In this study, we found that the exit threshold (g) was reduced in DPN rats and that this reduction could be reversed following lorazepam treatment, indicating that lorazepam can ameliorate mechanical allodynia in DPN rats.

Inflammation contributes to the progression of DPN [15] and numerous studies have focused on reducing inflammation to manage DPN. In this regard, acupuncture has been shown to modulate inflammation and slow the development of DPN [16]. Sinomenine has been found to decrease prostaglandin-

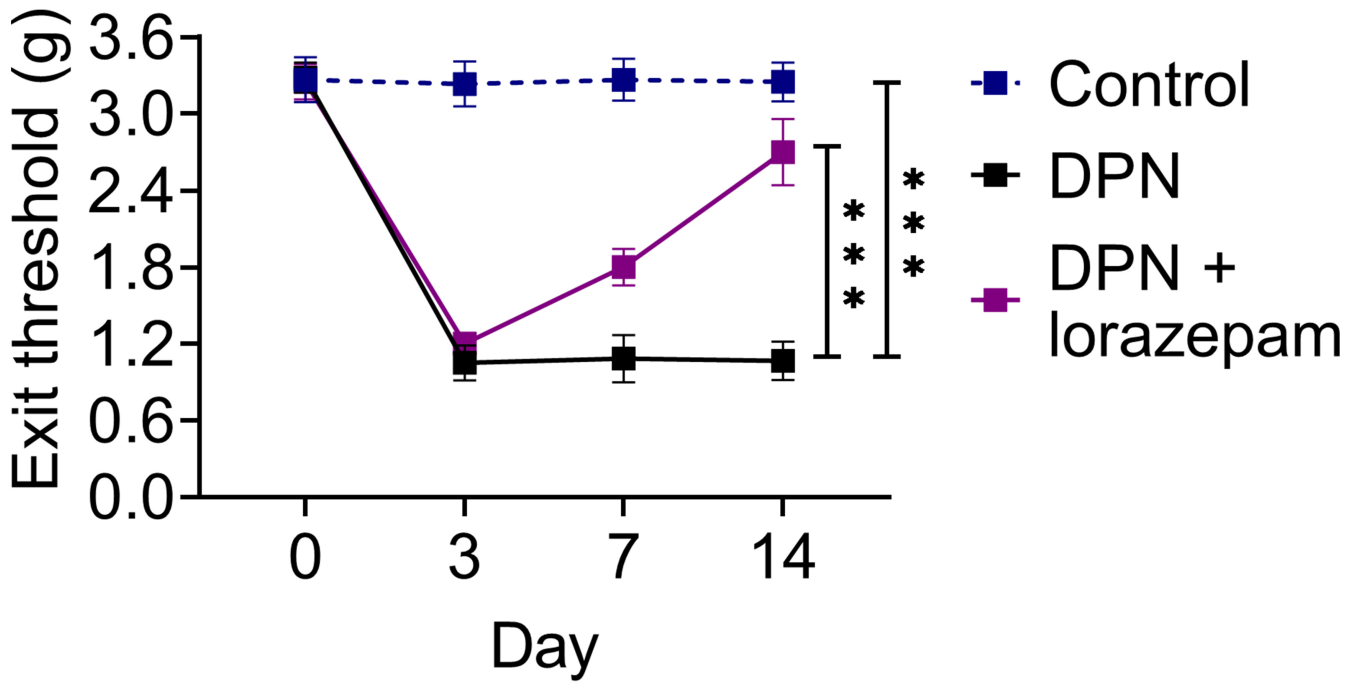

FIGURE 1. Lorazepam ameliorated mechanical allodynia in DPN rats. The investigated rats were assigned to a Control (n = 6), DPN (n = 6) and DPN + lorazepam (n = 6) group. Mechanical allodynia was assessed using the Von Frey test to determine withdrawal thresholds (g). The data are presented as mean ± SEM. ***$p < 0.001$. DPN: Diabetic peripheral neuropathy.

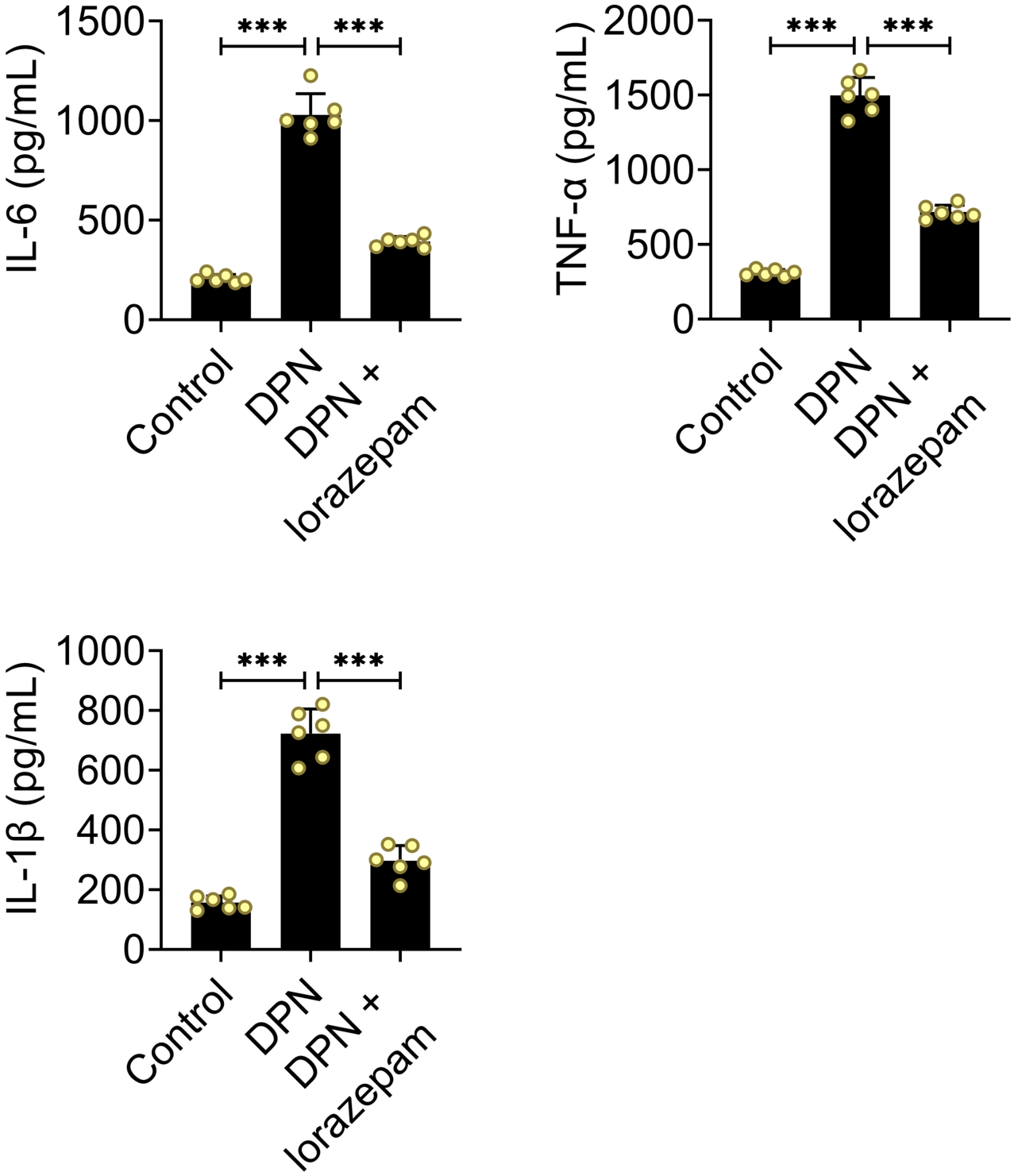

FIGURE 2. Lorazepam alleviated inflammation in DPN rats. Rats were categorized into Control (n = 6), DPN (n = 6), and DPN + lorazepam (n = 6) groups. Levels of TNF-α, IL-1β and IL-6 in serum were quantified using ELISA. The data are presented as mean ± SEM. ***$p < 0.001$. IL: interleukin; TNF-α: tumor necrosis factor-α; DPN: Diabetic peripheral neuropathy.

endoperoxide synthase 2 (PTGS2) expression, thereby inhibiting the inositol-requiring enzyme 1 alpha-X-box binding protein 1 pathway and improving DPN [17]. Additionally, curcumin reduced TNF-α expression, which helped alleviate DPN progression [18]. In this study, we observed that inflammation was elevated in DPN rats, but this increase was mitigated after lorazepam treatment.

The regulation of T cells can affect neuroinflammation. For instance, regulatory T cells ameliorate myelin loss and cognitive dysfunction by modulating the toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/NF-κB pathway [19]. Additionally, T cell infiltration regulates neuroinflammation in Alzheimer's disease [20]. T cells play a role in neurodegenerative diseases through their impact on neuroinflammation [21]. Furthermore, hippocampal T cell infiltration accelerates neuroinflammation in tauopathy mice

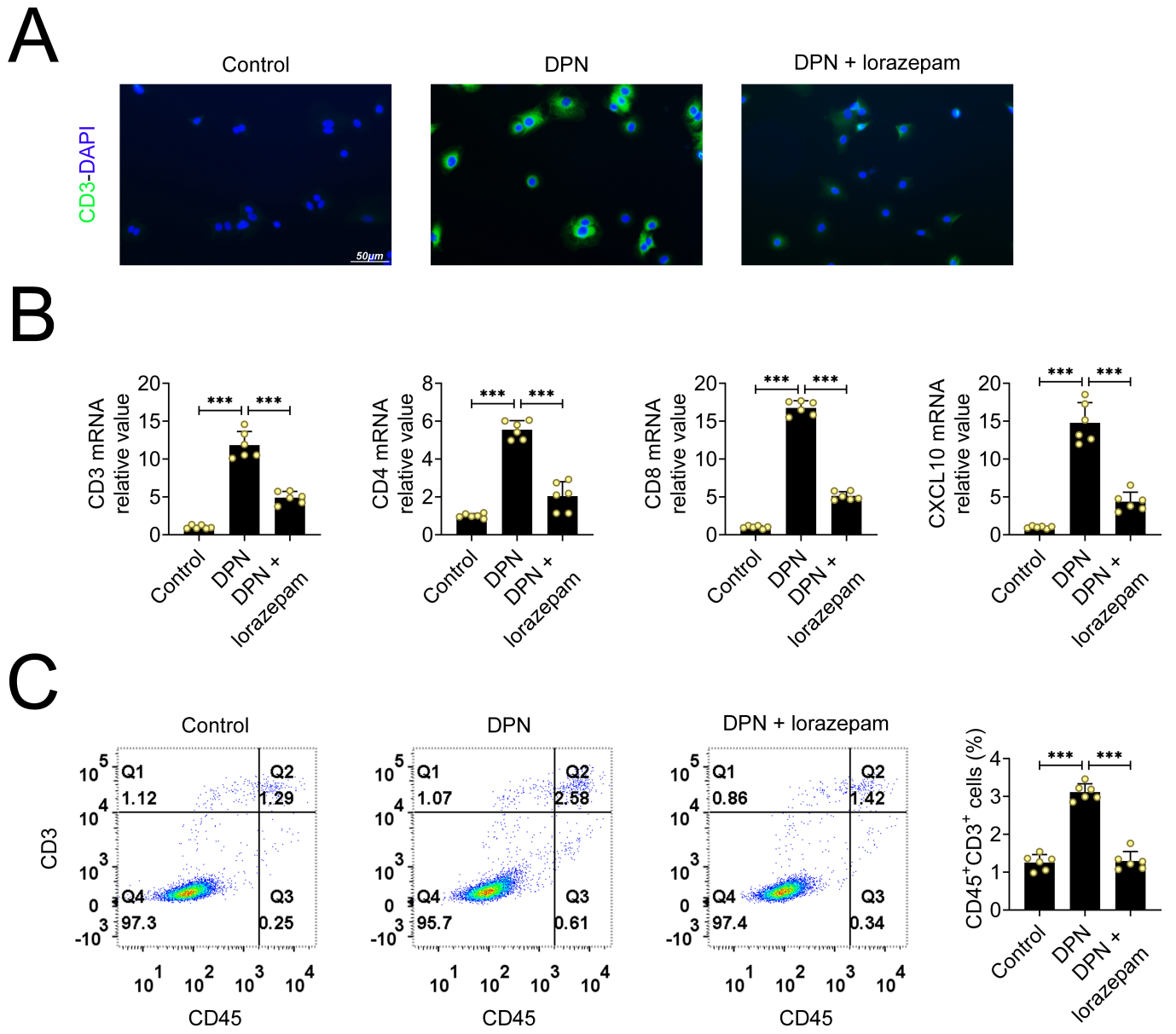

FIGURE 3. Lorazepam suppressed T cell infiltration in DPN rats. The rats were categorized into Control (n = 6), DPN (n = 6), and DPN + lorazepam (n = 6) groups. (A) CD3 levels in spinal cords (L4–6) were analyzed by IF assay. (B) mRNA expressions of *CD3*, *CD4*, *CD8* and *CXCL10* in spinal cords were measured using RT-qPCR. (C) The percentage of $CD45^+$ $CD3^+$ cells were assessed using flow cytometry. Data are presented as mean ± SEM. ***$p < 0.001$. DPN: Diabetic peripheral neuropathy; CD: Cluster of Differentiation; CXCL10: chemokine (C-X-C motif) ligand 10.

[22]. In this study, lorazepam was found to suppress T cell infiltration in DPN rats.

The NF-κB and Nrf2 pathways are essential in inflammation. For example, Hydnocarpin D modulates the NF-κB and Nrf2/HO-1 pathways to alleviate inflammation in lipopolysaccharide-stimulated acute lung injury [23]. Oxyberberine influences the Nrf2/NF-κB pathways to reduce inflammation and oxidative stress in 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-triggered colitis [24]. Allicin modulates the Nrf2 and NF-κB pathways to reduce inflammation in diabetic macroangiopathy [25]. Additionally, Nrf2 activates downstream genes such as HO-1 [26], an enzyme that regulates reactive oxygen species levels and possesses anti-inflammatory properties [27]. However, the regulatory impacts of lorazepam on the NF-κB and Nrf2 pathways in DPN remain unclear. In this study, it was revealed that the activated NF-κB and Nrf2 pathways in DPN rats were inhibited by lorazepam treatment.

5. Conclusions

In conclusion, lorazepam was found to reduce neuroinflammation by inhibiting T cell infiltration, thereby ameliorating DPN. This study has some limitations, and future research could include more experiments to investigate other regulatory functions of lorazepam in the development of DPN.

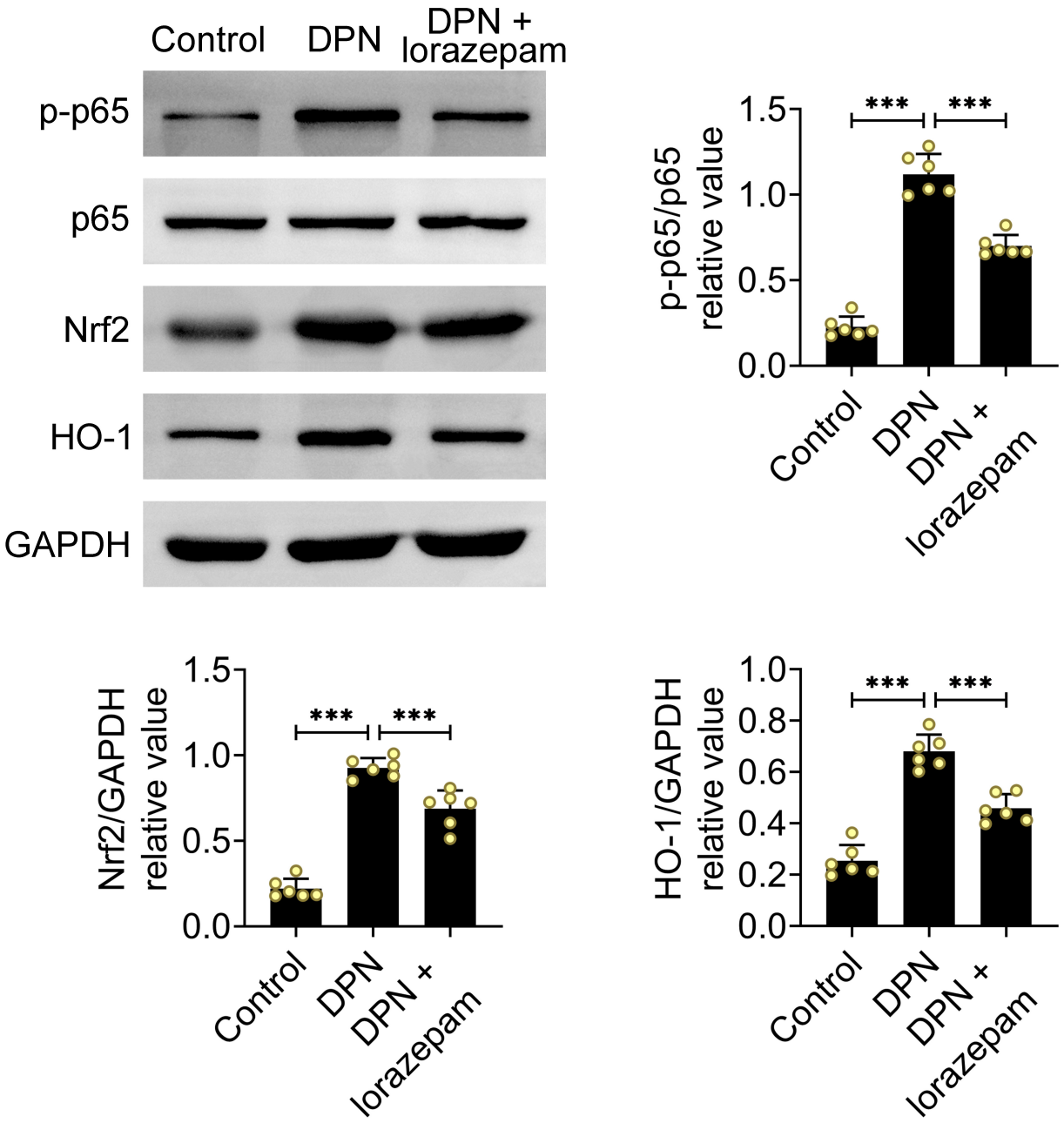

FIGURE 4. Lorazepam can obstruct the NF-κB and Nrf2 pathways. The investigated rats were categorized into Control (n = 6), DPN (n = 6), and DPN + lorazepam (n = 6) groups. Protein expressions of p-p65, p65, Nrf2 and HO-1 in spinal cords were analyzed by western blot. Data are presented as mean ± SEM. ***$p < 0.001$. DPN: Diabetic peripheral neuropathy; p-p65: phosphorylation-p65; Nrf2: Nuclear factor erythroid 2-related factor 2; HO-1: Heme Oxygenase-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YW, CLX—designed the study and carried them out. YW, YPS—supervised the data collection, analyzed the data, interpreted the data. YW, YPS, CLX—prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of People's Hospital of Lixia District of Jinan (Approval no. 2021033), and all animal experiments were conducted in accordance with the Care and Use of Laboratory Animals of the National Institutes of Health.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Ying Wang, Yaping Sui, Chunli Xing. Lorazepam attenuates neuroinflammation by suppressing T cell infiltration in diabetic peripheral neuropathy. Signa Vitae. 2024; 20(9): 72-78. doi: 10.22514/sv.2024.113.