Research Article

N-Acetylcysteine Attenuates Hyperalgesia in Rats with Diabetic Neuropathic Pain: Role of Oxidative Stress and Inflammatory Mediators and CXCR4

Sisi Li [1,2,3] **Xuying Li** [4] **Xiangbin Xie** [1] **Xiao Wei** [4] **Cong Yu** [3] **Chi Wai Cheung**,[2] **Zhengyuan Xia** [2] **and Guogang Tian** [1,4,5]

[1]*Department of Anesthesiology, Sanya Municipal Hospital, Sanya, China*
[2]*Department of Anesthesiology, The University of Hong Kong, Hong Kong, China*
[3]*Department of Anesthesiology, Stomatological Hospital of Chongqing Medical University, Chongqing, China*
[4]*Department of Emergency and Intensive Care Unit, The First Affiliated Hospital of Hainan Medical University, Haikou, China*
[5]*Key Laboratory of Emergency and Trauma of Ministry of Education, Hainan Medical University, Haikou, China*

Correspondence should be addressed to Zhengyuan Xia; zyxia@hku.hk and Guogang Tian; dr_tgg@126.com

Objectives. CXCR4 plays critical roles in the development of diabetic neuropathic pain (DNP) in rats, and its mechanism is unknown. This study was aimed at evaluating the potential therapeutic value of the antioxidant N-acetylcysteine (NAC) against DNP in rats and how CXCR4 participates in the formation of DNP. *Methods*. Control or streptozotocin- (STZ-) induced diabetic Sprague-Dawley rats received vehicle or NAC for four weeks starting one week after STZ injection. Von Frey and Hargreaves Apparatus were used to analyze the behavioral changes of mechanical allodynia and heat hyperalgesia. CXCR4, p-CXCR4, interleukin- (IL-) 6, and tumor necrosis factor- (TNF-) α in the spinal cord and the prefrontal cortex were detected by western blotting. Plasma IL-6, TNF-α, superoxide dismutase- (SOD-) 1, SOD-2, and lipid peroxidation products malondialdehyde (MDA) and 15-F2t-Isoprostane were detected by ELISA. *Results*. The values of paw withdrawal threshold (PWT) and paw withdrawal latencies (PWL) were reduced in diabetic rats compared to control rats that were concomitant with significant increases of CXCR4, p-CXCR4, IL-6, and TNF-α protein expressions in the spinal cord and prefrontal cortex. The treatment with NAC decreased the IL-6 and TNF-α protein expression and further increased CXCR4 and p-CXCR4 in the spinal cord and the cortex of diabetic rats that were accompanied with enhancement of PWT and PWL. NAC also significantly attenuated or reverted the increases of plasma IL-6, TNF-α, SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane in diabetic rats. *Conclusion*. It is concluded that NAC treatment could effectively alleviate DNP and that induction of CXCR4 and p-CXCR4 may represent a mechanism whereby NAC attenuates DNP.

1. Introduction

Diabetic neuropathic pain (DNP) is one of the most common chronic complications of diabetes, which brings suffering to diabetic patients [1, 2]. The underlying mechanisms of DNP are complex, and both peripheral and central components of the sensory systems are reported to be involved in the progression and maintenance of neuropathy. Increased oxidative stress is a unifying mechanism in the causation of DNP [3]. Hyperglycemia-induced activation of the polyol and hexosamine pathway and formation of advanced glycation end products are known to increase the production of Reactive Oxygen Species (ROS) that contribute to nerve injury [4, 5]. Excessive generation of ROS initiates a vicious circle by activating stress-sensitive pathways such as NF-Kb, p38 MAPK, and protein kinase C [6] or proinflammatory cytokines that contribute to diabetic complications [7, 8]. Hyperglycemia induces higher levels of proinflammatory cytokines that have been shown to correlate with the incidence of neuropathy [9, 10].
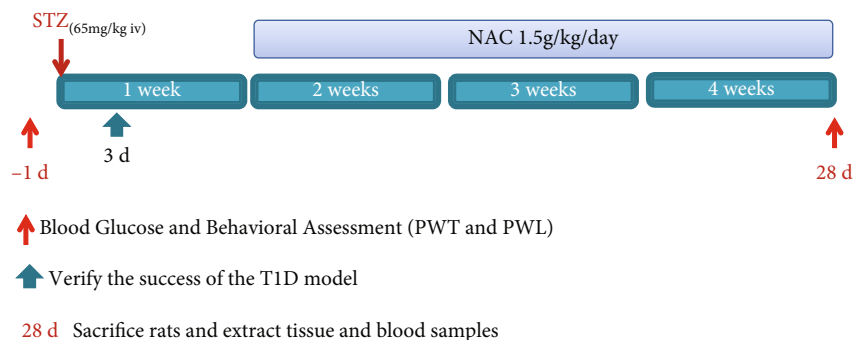
Figure 1: Schematic and timeline of the experimental design. PWT = paw withdrawal threshold; PWL = paw withdrawal latencies.

Chemokine receptor CXCR4 is a specific receptor for chemokine matrix-derived factor-1 (CXCL12). CXCR4/SDF-1 signaling has been implicated in the pathogenesis of neuropathic pain in a subset of animal models including diabetic neuropathic pain [11, 12]. A study showed that the specific CXCR4 antagonist AMD3100 can reverse DNP in rat models of type II diabetes and that CXCR4 coordinates inflammation in diabetic dorsal root ganglia [13] that could contribute to the development of pain in diabetes [14]. Our previous research found that the values of PWT and PWL were reduced in diabetic rats that were concomitant with significant increases of both CXCR4 and TNF-α protein expression in the DRG of diabetes [15]. However, the role of CXCR4 in relation to ROS in the development of DNP has yet to be determined.

NAC, a precursor of GSH, confers its antioxidant effect via enhancing endogenous GSH/GSSG [16]. NAC also produces a broad range of effects, which include attenuation of pain [17] and confer neuroprotection after spinal cord injury [18] and peripheral nerve lesions [19]. Our previous research found that expression of the prooxidative NADPH oxidase and inflammatory markers increased in the heart of STZ-induced diabetic rats, and these causes could be decreased by NAC [20, 21]. However, the potential effects and mechanism of NAC on DNP have not been explored.

Pain conduction is regulated by up- and downregulated pathways, which are caused by peripheral and central nervous system sensitization. Central sensitization is an important pathogenesis of neuropathic pain, and the maintenance of neuropathic pain lies in central sensitization [22]. Therefore, we proposed to observe the changes of inflammatory factors and CXCR4 protein expression in the spinal cord as well as the prefrontal cortex of diabetic rats with DNP and to determine the effects of antioxidant treatment with N-acetylcysteine on the inflammatory mediators and CXCR4 in the spinal cord and prefrontal cortex and the impact on diabetic neuropathic pain.

2. Materials and Methods

2.1. Experimental Animals.
Male adult Sprague-Dawley with streptozotocin- (STZ-) induced diabetes and age-matched non-diabetic control rats were used. All rats (250 ± 10 g, 6-8 weeks) were obtained from and housed in the Laboratory Animal Service Center (University of Hong Kong) and received standard care in accordance with the principles of Animal Care of the University of Hong Kong. The committee on the Use of Live Animals in Teaching and Research approved the experimental protocols.

2.2. Induction of Diabetes and Antioxidant Treatment.
Type 1 diabetes was induced by tail injection of STZ (Sigma, USA) at the dose of 65 mg/kg body weight in 0.1 mol/L citrate buffer (pH 4.5) or citrate buffer alone as control under anesthesia with a combination of ketamine 67.7 mg/kg body weight and xylazine 6.77 mg/kg body weight [23]. Blood glucose was tested by a OneTouch UltraVue glucometer (Johnson, USA) 72 hours after the injection. Rats with a blood glucose level higher than 16.7 mmol/L 72 hours at STZ injection were deemed T1DM and subjected to subsequent experiments. Blood glucose and body weight were measured at baseline and monitored weekly during the experiment.

Some of the diabetic rats were treated with NAC (Sigma-Aldrich, St. Louis, MO, USA). Rats were randomly divided into three groups of $n = 6$ per group: control (C), untreated diabetic rats (D), and diabetic rats treated with NAC (D+N). NAC (Sigma-Aldrich) was dissolved in drinking water for 3 weeks' duration of treatment starting 1 week after induction of diabetes (Figure 1). We used a dose of NAC at 1.5 g/kg/day; the optimal dosage was selected according to our previous experiment [24]. The daily dose of NAC (1.5 g/kg) was dissolved into 2/3 volume of the average daily amount of drinking water that was estimated based on preliminary study and previous studies to ensure that the total amount of NAC was taken by the rats before additional amount of water was supplied. Meanwhile, the plasma glucose, body weight, and water intake of rats were routinely monitored.

2.3. Behavioral Assessment.
Rats were subjected to behavioral assessments before the STZ injection (baseline) and at four weeks after STZ injection. To measure mechanical sensitivity, paw withdrawal threshold (PWT) of rats was assessed by the electronic Von Frey test (IITC) [25]. Before the experiments, the rats were placed in a transparent plastic dome with a metal mesh floor for nearly 30 minutes. The electronic Von Frey filament set has a calibrated range of bending force (floating 1~90 g) to record automatically pain threshold. A single filament was applied to the plantar surface 5 times with an interstimulation interval of 5 seconds. A positive response was defined as at least 1 clear withdrawal response in the 5
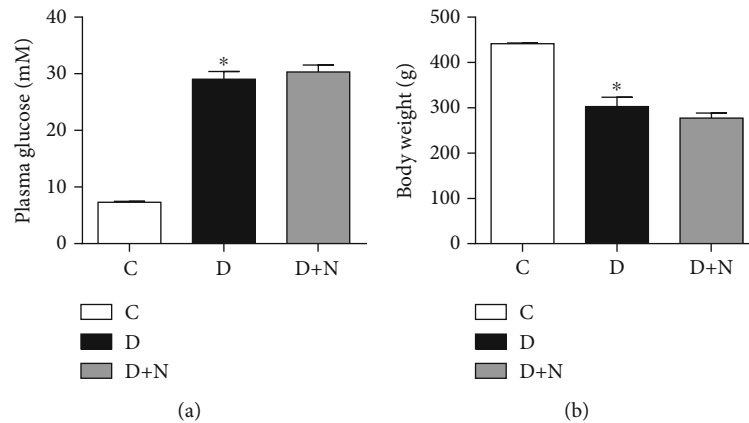
applications. Licking of the paw was also considered as a positive response.

To quantitatively assess the thermal threshold of the hind paw, rats were placed on the glass surface of a thermal testing apparatus with acclimatization for 30 minutes before testing. A mobile radiant heat source (Hargreaves apparatus) located under the glass was focused onto each of both hind paws of each rat. A radiant heat source was applied individually to the middle of either hind paw for up to 60 seconds, with the paw withdrawal threshold (PWT) measured. The heating rate ramped from 30°C to 58°C over 60 seconds in a consistent fashion on each occasion [26]. The cut-off of 30 seconds was used to prevent potential tissue damage. The paws were inspected before and after thermal testing to ensure that no evidence of thermal damage was present. The withdrawal latency of both hind paws from three consecutive trials was averaged, and the mean value was used as the thermal threshold. There were 15-minute intervals in between trials.

2.4. Western Blot Analysis. After the completion of behavioral assessment, rats were then deeply anaesthetized with sodium pentobarbital (65 mg/kg), and the lumbar (L3 to L5) parts of the spinal cord and the prefrontal cortex were rapidly excised and frozen in liquid nitrogen. The tissues were homogenized using lysis buffer, sonicated, and centrifuged at 12000g for 20 min at 4°C. The protein concentration was quantified by bovine serum albumin and measured with the absorption of Coomassie brilliant blue in the spectrophotometer. Thereafter, the samples were frozen at -20°C for later use. Proteins were assessed by standard western blotting as described [27]. Briefly, equal quantities of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were blocked in 5% nonfat dry milk diluted with Tris-Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, pH 7.5, 0.1% Tween 20) at room temperature for 1 hour (h) and then probed with antibodies against TNF-α, IL-6, (Abcam, England), CXCR4, and p-CXCR4 (Sigma, USA) at 4°C overnight. After extensive washing, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies

(diluted 1 : 2000; Amersham Biosciences, UK). The immunoblots were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein bands were observed by an Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and then processed by gray scanning using ImageJ (National Institutes of Health, USA). All the target proteins were normalized by β-tubulin and calculated as percentage of the control.

2.5. Plasma TNF-α, IL-6, SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane Measurement. The blood samples were extracted from the heart of rats. Plasma samples were immediately separated by centrifugation at 3000 rpm for 15 min at 4°C and then divided into aliquots and stored at −80°C for subsequent assays. Plasma TNF-α, IL-6, SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane activity was detected using commercially available kits (Cayman Chemical) as described previously [23, 28, 29].

2.6. Statistical Analysis. All values are presented as means ± SD. One-way analysis of variance (ANOVA) was used for statistical analysis (GraphPad Software, Inc.) of data obtained within the same group and between groups, respectively, followed by Tukey's test for multiple comparisons of group means. P values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. General Characteristics. The baseline body weight and blood glucose did not significantly differ among rats in the three experimental groups (data not shown). As shown in Figure 2, STZ-injected rats had significant diabetic symptoms of hyperglycemia and weight loss. The blood glucose level of the diabetic rats increased but their body weight decreased compared to normal rats ($^{*}P < 0.05$, D vs. C). NAC treatment for three weeks had no significant effect on body weight and blood glucose in diabetic rats ($P > 0.05$, D+N vs. D).

3.2. Effect of N-Acetylcysteine Treatment on Behavioral Parameters in Diabetic Rats. Baseline values of paw
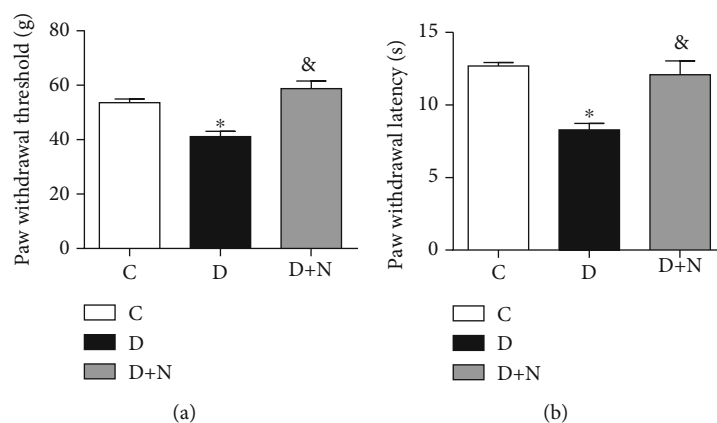
(a)

(b)

FIGURE 3: Variations of (a) PWT and (b) PWL during the progression of STZ-induced diabetes (D) and treated with N-acetylcysteine (D+N) in rats. All results are presented as means ± SD, $n = 6$/group; *$P < 0.05$ (D vs. C), &$P < 0.05$ (D+N vs. D).

withdrawal latencies (PWL) and paw withdrawal threshold (PWT) were tested in rats in the three groups, and no significant difference existed between the control and diabetes groups.

The values of PWT were significantly lower in the diabetes group up to 4 weeks of diabetes than those in the control group (*$P < 0.05$, Figure 3(a)). At week 4 post STZ injection, the administration of NAC attenuated mechanical hyperalgesia (&$P < 0.05$, Figure 3(a)).

A significant decrease in the PWL was observed 4 weeks in diabetes rats than in the control group (*$P < 0.05$, Figure 3(b)). At week 4 post STZ injection, the administration of NAC attenuated thermal hyperalgesia (&$P < 0.05$, Figure 3(b)).

3.3. Effect of N-Acetylcysteine Treatment on TNF-α and IL-6 Protein Expression in the Spinal Cord and Prefrontal Cortex of Diabetic Rats. As shown in Figure 4, significant increases in TNF-α and IL-6 were observed in diabetic rats compared to nondiabetic rats both in the spinal cord and in the prefrontal cortex (*$P < 0.05$, D vs. C). NAC treatment significantly decreased the proinflammation TNF-α and IL-6 levels compared to diabetic rats (&$P < 0.05$, D+N vs. D).

3.4. Effect of N-Acetylcysteine Treatment on CXCR4 and p-CXCR4 Protein Expression in the Spinal Cord and the Prefrontal Cortex of Diabetic Rats. As shown in Figure 5, significant increases in CXCR4 and p-CXCR4 were observed in diabetic rats compared to nondiabetic rats both in the spinal cord and prefrontal cortex (*$P < 0.05$, D vs. C). NAC treatment further significantly increased the CXCR4 and p-CXCR4 levels compared to those in diabetic rats (&$P < 0.05$, D+N vs. D).

3.5. Effect of N-Acetylcysteine Treatment on Plasma IL-6 and TNF-α Level of Diabetic Rats. As shown in Figure 6, significant increases in plasma IL-6 and TNF-α were observed in diabetic rats as compared to nondiabetic rats (*$P < 0.05$, D vs. C). NAC treatment significantly decreased the plasma IL-6 and TNF-α levels compared to diabetic rats (&$P < 0.05$, D+N vs. D).

3.6. Effect of N-Acetylcysteine Treatment on Plasma SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane Level of Diabetic Rats. As shown in Figure 7, significant increases in the

plasma SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane were observed in diabetic rats compared to nondiabetic rats (*$P < 0.05$, D vs. C). NAC treatment significantly decreased the plasma SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane compared to those in diabetic rats (&$P < 0.05$, D+N vs. D).

4. Discussion

In the present study, firstly, we found that the pain threshold decreased significantly after the establishment of diabetes. Furthermore, proinflammatory proteins TNF-α and IL-6 were significantly upregulated in the spinal cord, cortex, and plasma. Plasma SOD-1 and SOD-2 activities in diabetic rats also increased significantly as compared to those in control rats. CXCR4 and p-CXCR4 expression was significantly increased both in the spinal cord and cortex. Thirdly, NAC treatment could increase the pain threshold, at the same time, downregulate the expression of TNF-α and IL-6, and upregulate CXCR4 and p-CXCR4 expression, compared to the diabetic rats.

STZ impairs β-cell in the pancreas, and intravenous STZ at the dose of 65 mg/kg reliably and reproducibly induced type 1 diabetes in rats [30, 31]. Hyperglycemia induced increases in the production of Reactive Oxygen Species (ROS) that contribute to nerve injury [32]. Excessive oxidative stress and overload of $Ca^{(2+)}$ entry are common features of neuropathic pain. In whole-cell patch clamp experiments, $TRPM_2$ currents in the DRG following diabetes induction with STZ were gated by H_2O_2 and H_2O_2-induced TRPM2 gating was totally inhibited by NAC [33]. NAC may have a protective role on oxidative stress and calcium influx through regulation of the $TRPM_2$ channel in diabetic neurons. A study showed that NAC, given after the establishment of diabetes, may offer protection against the risk for stroke via enhancing platelet GSH and GSH-dependent methylglyoxal elimination and SOD-1 [34]. Ninety patients with DNP completed the eight-week course of the study, and the results showed that NAC significantly increased serum levels of SOD and glutathione peroxidase 1 and alleviated painful symptoms of diabetic neuropathy [35].

Our current research found that plasma SOD-1 and SOD-2 activities were increased in diabetic rats as compared
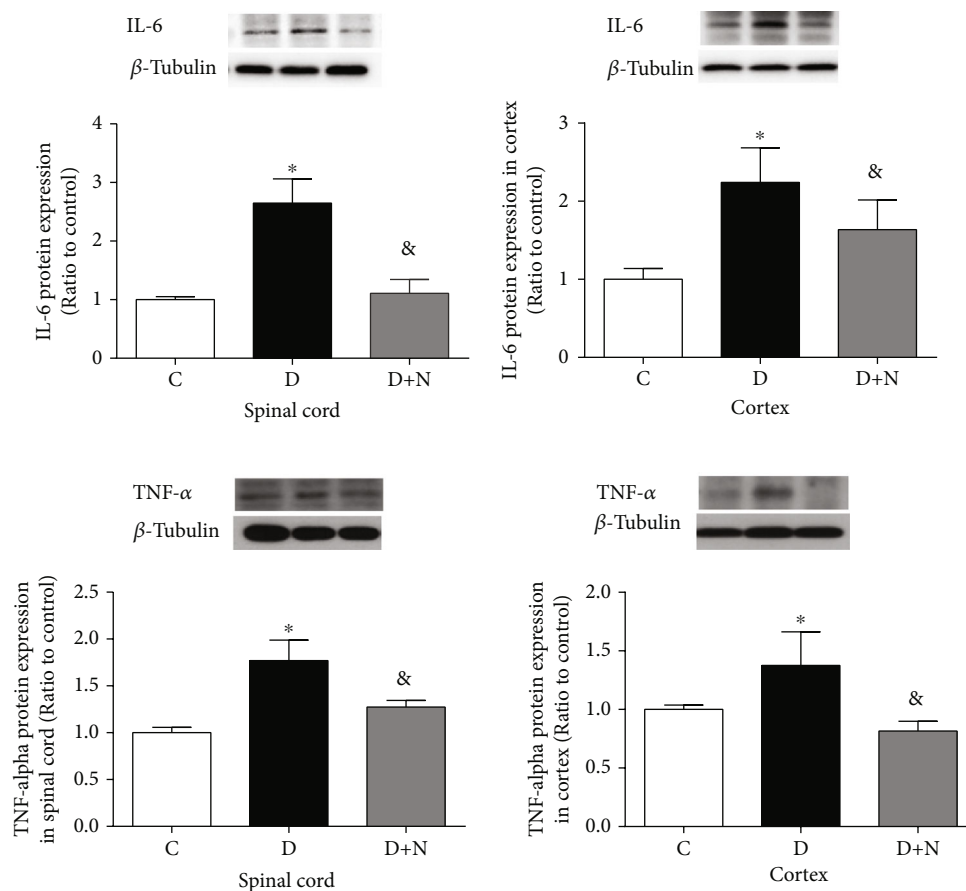
FIGURE 4: Effect of three weeks of NAC treatment on change in expression of IL-6 and TNF-α in the spinal cord and prefrontal cortex of diabetic rats measured by western blotting analysis. All results are presented as means ± SD, $n = 6$/group; $^*P < 0.05$ (D vs. C), $^\&P < 0.05$ (D +N vs. D).

to nondiabetic rats. These might be the consequence of hyperglycemia-induced overreaction of the oxidative stress system (SOD-1 and SOD-2) as a compensative response against oxidative stress, one of the key mechanisms of DNP. NAC increased pain threshold, and in the meantime, the effectiveness of NAC antioxidant therapy may have served to attenuate the compensative increases of SOD activities. The effectiveness of NAC antioxidant therapy may also be the major mechanism that resulted in overexpression of CXCR4 and p-CXCR4 in the spinal cord and cortex.

In the state of hyperglycemia, oxidases in various compartments, including the endothelium, media, and adventitia, produce ROS which render vascular smooth muscle cells (VSMCs) under oxidative stress [36]. The oxidative stress may injure VSMC or perturb the regulatory signal to induce the upregulation of inflammatory factors [37]. Our previous research found that IL-6 and TNF-α were increased in the plasma and the myocardium of STZ-induced diabetic rats [38]. The patients with diabetic kidney disease (DKD) showed higher IL-6 and TNF-α level than those without DKD [39]. The levels of IL-6 and TNF-α in the livers [40] and the spinal cord [41] of type I diabetic rats were also increased as compared to those of normal rats. There is an important discovery in our current experiment that IL-6 and TNF-α increased significantly not only in the spinal cord

and plasma but also in the cortex. This suggests that the inflammatory response in the peripheral and central systems together leads to the DNP.

In our study, NAC significantly attenuated the increases of IL-6 and TNF-α levels in diabetic rats in the spinal cord, cortex, and plasma. In clinic, NAC is efficacious in improving neuropathic pain associated with diabetic neuropathy [35]. This further proved the important mechanism of DNP that hyperglycemia produced too much ROS with the subsequent presence of strong oxidative stress that induces acute inflammatory response.

CXCR4 is a G protein-coupled receptor that is involved in homing and chemotaxis in the hematopoietic and immune systems. The ligand of CXCR4 is a stromal cell-derived factor (SDF)-1/CXCL12 that is associated with stem cell migration from the bone marrow to the cells expressing SDF-1 [42]. Intrathecal administration of SDF-1 induced hypersensitivity in naive rats. And repeated intrathecal administration of the CXCR4 antagonist, AMD3100, significantly suppressed the initiation and duration of neuropathic pain [11, 43]. SDF-1/CXCR4 signaling also maintains central poststroke pain [44] and persistent abdominal pain in rats with chronic pancreatitis [45]. SDF-1/CXCR4 signaling contributes to bone cancer pain [46] and diabetic neuropathic pain [15, 47].
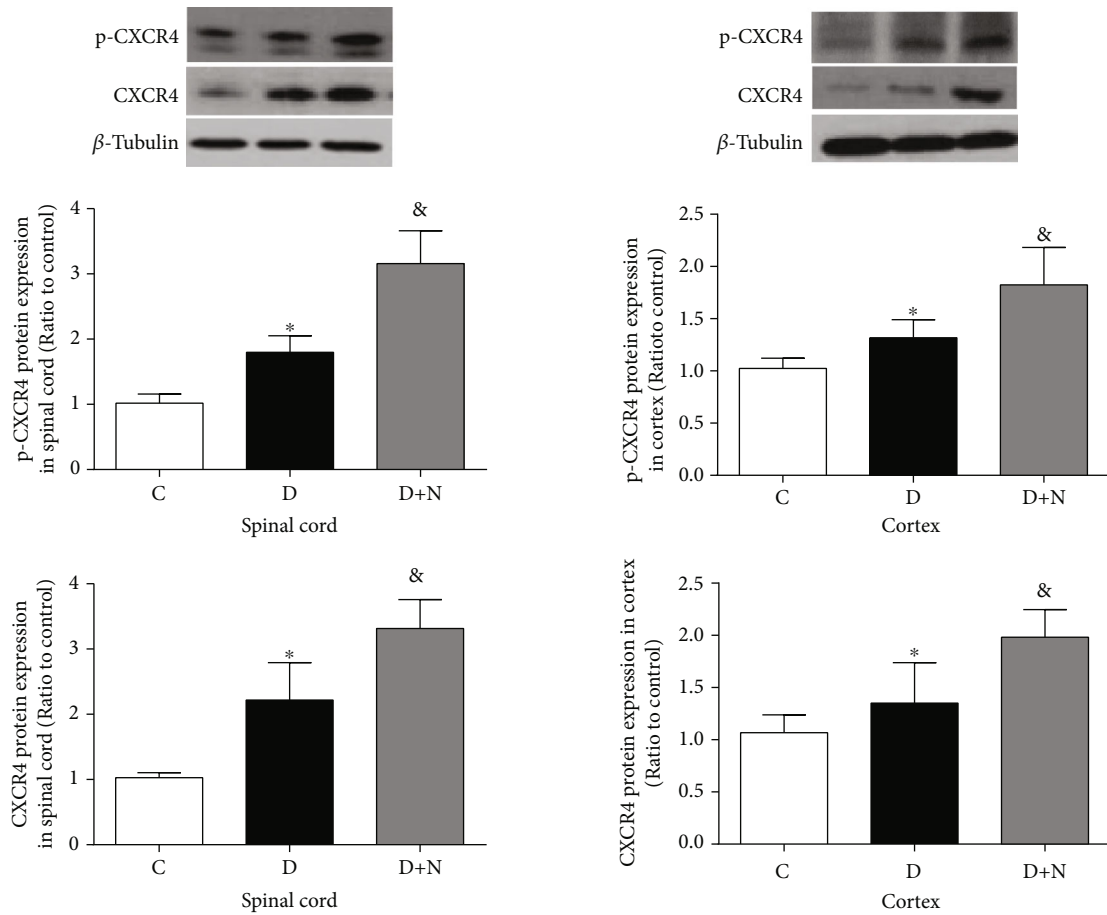
FIGURE 5: Effect of three weeks of NAC treatment on change in expression of CXCR4 and p-CXCR4 in the spinal cord and prefrontal cortex of diabetic rats measured by western blotting analysis. All results are presented as means ± SD, $n = 6$/group; $^*P < 0.05$ (D vs. C), $^\&P < 0.05$ (D+N vs. D).
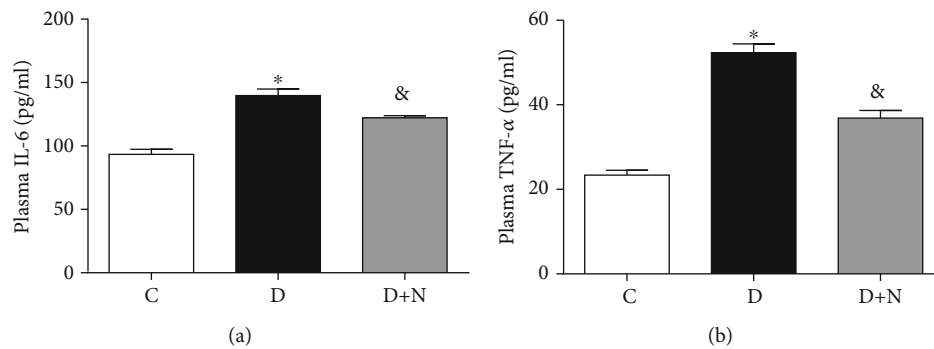

(a)

(b)

FIGURE 6: Effect of three weeks of NAC treatment on change in plasma IL-6 and TNF-α level of diabetic rats measured by ELISA analysis. All results are presented as means ± SD, $n = 6$/group; $^*P < 0.05$ (D vs. C), $^\&P < 0.05$ (D+N vs. D).

In our study, CXCR4 and p-CXCR4 receptor expression increased significantly in the spinal cord and cortex in diabetic rats. This indicated that CXCR4 and p-CXCR4 overexpression may have contributed to the development of DNP in the absence of antioxidant treatment. However, what our study found especially interesting was that NAC treatment relieves DNP but further increases CXCR4 and p-CXCR4 protein expression both in the spinal cord and cortex. This

result seems contradictory to what has been reported about a detrimental role of CXCR4 in pain development. It should be noted, however, among the chemokine receptors, CXCR4 stands out for its pleiotropic role as well as for its involvement in normal and abnormal development, including immune diseases, viral infections, and cancer [48]. SDF-1 can activate CXCR4-expressing cells and induce B cell proliferation and T cell recruitment. CXCR4$^{-/-}$ mice display
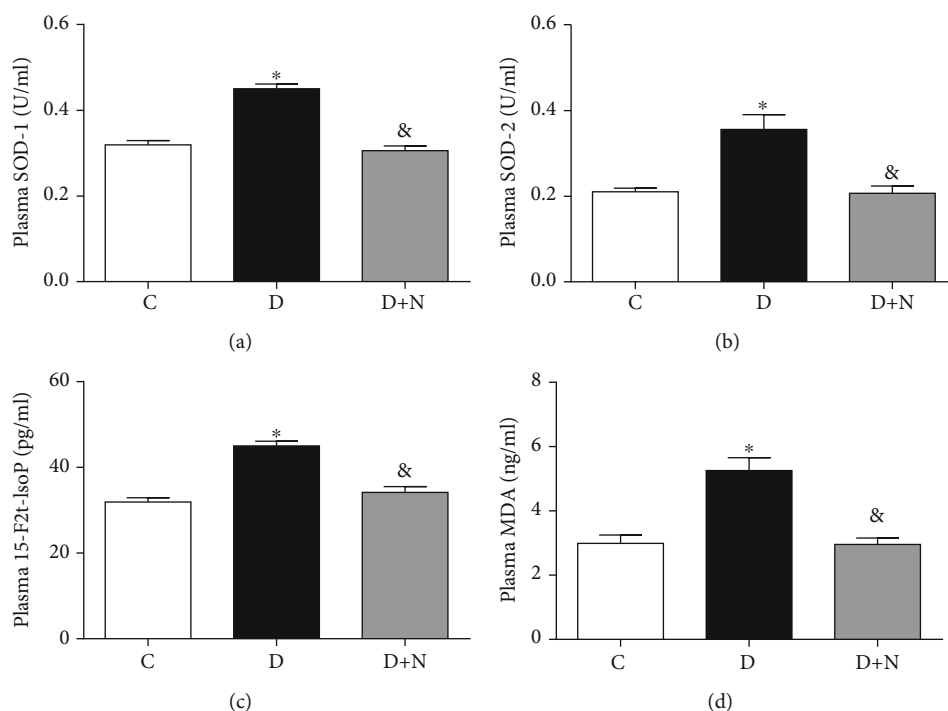
FIGURE 7: Effect of three weeks of NAC treatment on change in plasma SOD-1, SOD-2, MDA, and 15-F2t-Isoprostane level of diabetic rats measured by ELISA analysis. All results are presented as means ± SD, $n = 6$/group; $^*P < 0.05$ (D vs. C), $^\&P < 0.05$ (D+N vs. D).

impaired vascularization in various organs, including the intestines, stomach, and heart [49]. A study using knockout mice has revealed the involvement of CXCR4 in cardiogenesis and brain development. Without CXCR4, the homozygous knockout mice die before birth. In an in vivo study, CXCR4-overexpressing MSCs were observed with reduced retinal damage and upregulation of rhodopsin and NSE protein and downregulation of inflammatory cytokines IL-6 and TNF-α [50]. Therefore, CXCR4 is a key molecule for normal development.

A study showed that ROS accumulation in prostate cancer cell lines resulted from activation of the NADPH oxidase (NOX) family of enzymes. NOX2 responded to and was regulated by the SDF-1α/CXCR4 signaling axis [51]. The mice treated with NAC presented markedly activated GSH-PX and SOD in mice with crystalline silica-induced pulmonary fibrosis and at the same time downregulation of oxidizing enzymes (NOX2, iNOS, SOD-2, and XO) [52]. In our study, three weeks of 1.5 g/kg NAC in water treatment reduced oxidative stress. It is possible that NAC treatment may have resulted in a downregulation of NOX2 and the reduction of oxidative stress, as a consequence of the upregulation of CXCR4 and p-CXCR4 expression. Thus, it is intriguing to hypothesize that increasing CXCR4 and p-CXCR4 expression may represent one of the major mechanisms whereby NAC attenuates DNP, although this hypothesis needed to be further tested.

There are clinical study findings showing beneficial effects of enhancing CXCR4 in diabetic patients. Diabetic Foot Ulcer (DFU) is the most common in patients who have diabetic peripheral neuropathy and angiopathy as well as a foot deformity. Diabetic foot early performance is mainly for cold foot, numbness, and other abnormal feeling and later will appear sensory decline or loss. Research showed that SDF-1, a specific ligand of CXCR4, was significantly decreased in both the circulation and tissue biopsies of patients with T2DM and infected DFU [53]. Contents and protein expressions of SDF-1α/CXCR4 protein expression in wound tissue of diabetic patients with DFU who received 8 weeks treatment of a Chinese ointment (Xiaodu Yuji Paste) were all significantly higher than those in the control group that was accompanied with improved angiogenesis and wound healing [54]. Therefore, we speculate that the increased expression of CXCR4 and p-CXCR4 in the early phase of diabetes as seen in our current study (e.g., 4 weeks after STZ induction) would protect blood vessels, but in the meantime may lead to abnormal nerve conduction, which eventually results in the decrease of pain threshold. Another study suggests that age-associated reduction in CXCR4 expression on BM MSC impairs hematopoietic niche activity with increased ROS production, driving an HSC aging phenotype. Thus, modulation of the SDF-1/CXCR4 axis in MSC by N-acetyl-L-cysteine (NAC) may improve their niche supporting activity and attenuate the HSPC aging phenotype [55]. The SDF-1/CXCR4 pathway plays an important role in pain conduction. Therefore, we speculate that hyperalgesia caused by the increased expression of CXCR4 in the early stage of diabetes is a protective mechanism. The CXCR4 inhibitor AMD3100 alleviates hyperalgesia by reducing pain transmission pathways. NAC, as an antioxidant, further increased the expression of CXCR4 and alleviated hyperalgesia through protective pathway. The exact mechanism of CXCR4 in the development of diabetic neuropathic pain merits further in-depth studies.

In conclusion, in our current experimental setting, enhancement of CXCR4 and p-CXCR4 by NAC treatment was associated with the attenuation of DNP. This intriguing finding merits further studies to elucidate the role of CXCR4 in the development of pain in general and in DNP specifically.

Data Availability

Research data will be available from corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Sisi Li and Xuying Li contributed equally in this study.

Acknowledgments

References

[1] C. Toth, J. Lander, and S. Wiebe, "The prevalence and impact of chronic pain with neuropathic pain symptoms in the general population," *Pain Medicine*, vol. 10, no. 5, pp. 918–929, 2009.

[2] Y.-C. Weng, S.-S. Tsai, R.-K. Lyu et al., "Diabetic distal symmetrical polyneuropathy: correlation of clinical, laboratory, and electrophysiologic studies in patients with type 2 diabetes mellitus," *Journal Diabetes Research*, vol. 2020, article 6356459, pp. 1–11, 2020.

[3] A. M. Vincent, J. L. Edwards, L. L. McLean et al., "Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy," *Acta Neuropathologica*, vol. 120, no. 4, pp. 477–489, 2010.

[4] J. Johansen, A. K. Harris, D. J. Rychly, and A. Ergul, "Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice," *Cardiovascular Diabetology*, vol. 4, no. 1, p. 5, 2005.

[5] A. P. Rolo and C. M. Palmeira, "Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress," *Toxicology and Applied Pharmacology*, vol. 212, no. 2, pp. 167–178, 2006.

[6] S. Li, J. Zeng, X. Wan et al., "Enhancement of spinal dorsal horn neuron NMDA receptor phosphorylation as the mechanism of remifentanil induced hyperalgesia: roles of PKC and CaMKII," *Molecular Pain*, vol. 13, 2016.

[7] D. Ziegler, "Treatment of diabetic neuropathy and neuropathic pain: how far have we come?," *Diabetes Care*, vol. 31, Supplement 2, pp. S255–S261, 2008.

[8] F. Fiordaliso, A. Leri, D. Cesselli et al., "Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death," *Diabetes*, vol. 50, no. 10, pp. 2363–2375, 2001.

[9] L. Leung and C. M. Cahill, "TNF-α and neuropathic pain - a review," *Journal of Neuroinflammation*, vol. 7, no. 1, p. 27, 2010.

[10] R. B. Goldberg, "Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications," *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 9, pp. 3171–3182, 2009.

[11] Z. Y. Liu, Z. W. Song, S. W. Guo et al., "CXCL12/CXCR4 signaling contributes to neuropathic pain via central sensitization mechanisms in a rat spinal nerve ligation model," *CNS Neuroscience & Therapeutics*, vol. 25, no. 9, pp. 922–936, 2019.

[12] C. A. da Silva Junior, C. J. de Castro Junior, E. M. R. Pereira et al., "The inhibitory effect of Phα1β toxin on diabetic neuropathic pain involves the CXCR4 chemokine receptor," *Pharmacological Reports*, vol. 72, no. 1, pp. 47–54, 2020.

[13] C. Wang, W. Yuan, A. Hu et al., "Dexmedetomidine alleviated sepsis-induced myocardial ferroptosis and septic heart injury," *Molecular Medicine Reports*, vol. 22, no. 1, pp. 175–184, 2020.

[14] D. M. Menichella, B. Abdelhak, D. Ren, A. Shum, C. Frietag, and R. J. Miller, "CXCR4 chemokine receptor signaling mediates pain in diabetic neuropathy," *Molecular Pain*, vol. 10, article 1744-8069-10-42, 2014.

[15] D. Zhu, T. Fan, X. Huo, J. Cui, C. W. Cheung, and Z. Xia, "Progressive increase of inflammatory CXCR4 and TNF-alpha in the dorsal root ganglia and spinal cord maintains peripheral and central sensitization to diabetic neuropathic pain in rats," *Mediators of Inflammation*, vol. 2019, Article ID 4856156, 2019.

[16] G. Aldini, A. Altomare, G. Baron et al., "N-Acetylcysteine as an antioxidant and disulfide breaking agent: the reasons why," *Free Radical Research*, vol. 52, no. 7, pp. 751–762, 2018.

[17] A. K. Naik, S. K. Tandan, S. P. Dudhgaonkar et al., "Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats," *European Journal of Pain*, vol. 10, no. 7, pp. 573–579, 2006.

[18] A. Karalija, L. N. Novikova, P. J. Kingham, M. Wiberg, and L. N. Novikov, "Neuroprotective effects of N-acetyl-cysteine and acetyl-L-carnitine after spinal cord injury in adult rats," *PLoS One*, vol. 7, no. 7, article e41086, 2012.

[19] D. Welin, L. N. Novikova, M. Wiberg, J. O. Kellerth, and L. N. Novikov, "Effects of N-acetyl-cysteine on the survival and regeneration of sural sensory neurons in adult rats," *Brain Research*, vol. 1287, pp. 58–66, 2009.

[20] Z. Guo, Z. Xia, J. Jiang, and J. H. McNeill, "Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 292, no. 4, pp. H1728–H1736, 2007.

[21] G. Y. Tsai, J. Z. Cui, H. Syed et al., "Effect of N-acetylcysteine on the early expression of inflammatory markers in the retina and plasma of diabetic rats," *Clinical & Experimental Ophthalmology*, vol. 37, no. 2, pp. 223–231, 2009.

[22] J. N. Campbell and R. A. Meyer, "Mechanisms of neuropathic pain," *Neuron*, vol. 52, no. 1, pp. 77–92, 2006.

[23] T. Wang, S. Qiao, S. Lei et al., "N-Acetylcysteine and allopurinol synergistically enhance cardiac adiponectin content and reduce myocardial reperfusion injury in diabetic rats," *PLoS One*, vol. 6, no. 8, article e23967, 2011.

[24] X. Mao, T. Wang, Y. Liu et al., "N-Acetylcysteine and allopurinol confer synergy in attenuating myocardial ischemia injury via restoring HIF-1α/HO-1 signaling in diabetic rats," *PLoS One*, vol. 8, no. 7, article e68949, 2013.

[25] V. K. L. Hung, S. M. Y. Chen, L. W. Tai, A. Y. S. Chen, S. K. Chung, and C. W. Cheung, "Over-expression of endothelin-1

in astrocytes, but not endothelial cells, ameliorates inflammatory pain response after formalin injection," *Life Sciences*, vol. 91, no. 13-14, pp. 618–622, 2012.

[26] C. C. Toth, N. M. Jedrzejewski, C. L. Ellis, and W. H. Frey, "Cannabinoid-mediated modulation of neuropathic pain and microglial accumulation in a model of murine type I diabetic peripheral neuropathic pain," *Molecular Pain*, vol. 6, pp. 1744–8069, 2010.

[27] H. Li, W. Yao, M. G. Irwin et al., "Adiponectin ameliorates hyperglycemia-induced cardiac hypertrophy and dysfunction by concomitantly activating Nrf2 and Brg1," *Free Radical Biology & Medicine*, vol. 84, pp. 311–321, 2015.

[28] J. Luo, D. Yan, S. Li et al., "Allopurinol reduces oxidative stress and activates Nrf2/p62 to attenuate diabetic cardiomyopathy in rats," *Journal of Cellular and Molecular Medicine*, vol. 24, no. 2, pp. 1760–1773, 2019.

[29] J. Xu, S. Lei, Y. Liu et al., "Antioxidant N-acetylcysteine attenuates the reduction of Brg1 protein expression in the myocardium of type 1 diabetic rats," *Journal Diabetes Research*, vol. 2013, article 716219, pp. 1–8, 2013.

[30] T. Szkudelski, "The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas," *Physiological Research*, vol. 50, no. 6, pp. 537–546, 2001.

[31] L. W. Tai, Z. Pan, L. Sun et al., "Suppression of Pax2 attenuates allodynia and hyperalgesia through ET-1-ETAR- NFAT5 signaling in a rat model of neuropathic pain," *Neuroscience*, vol. 384, pp. 139–151, 2018.

[32] I. Jialal, S. Devaraj, and S. K. Venugopal, "Oxidative stress, inflammation, and diabetic vasculopathies: the role of alpha tocopherol therapy," *Free Radical Research*, vol. 36, no. 12, pp. 1331–1336, 2009.

[33] E. Sozbir and M. Naziroglu, "Diabetes enhances oxidative stress-induced TRPM2 channel activity and its control by N-acetylcysteine in rat dorsal root ganglion and brain," *Metabolic Brain Disease*, vol. 31, no. 2, pp. 385–393, 2016.

[34] B. Wang, T. Yee Aw, and K. Y. Stokes, "N-Acetylcysteine attenuates systemic platelet activation and cerebral vessel thrombosis in diabetes," *Redox Biology*, vol. 14, pp. 218–228, 2018.

[35] N. Heidari, F. Sajedi, Y. Mohammadi, M. Mirjalili, and M. Mehrpooya, "Ameliorative effects of N-acetylcysteine as adjunct therapy on symptoms of painful diabetic neuropathy," *Journal of Pain Research*, vol. Volume 12, pp. 3147–3159, 2019.

[36] C. P. Lin, P. H. Huang, C. F. Lai, J. W. Chen, S. J. Lin, and J. S. Chen, "Simvastatin attenuates oxidative stress, NF-κB activation, and artery calcification in LDLR-/- mice fed with high fat diet via down-regulation of tumor necrosis factor-α and TNF receptor 1," *PLoS One*, vol. 10, no. 12, article e0143686, 2015.

[37] C. F. Lai, J. S. Shao, A. Behrmann, K. Krchma, S. L. Cheng, and D. A. Towler, "TNFR1-activated reactive oxidative species signals up-regulate osteogenic Msx2 programs in aortic myofibroblasts," *Endocrinology*, vol. 153, no. 8, pp. 3897–3910, 2012.

[38] Z. Guo, Z. Xia, V. G. Yuen, and J. H. McNeill, "Cardiac expression of adiponectin and its receptors in streptozotocin-induced diabetic rats," *Metabolism*, vol. 56, no. 10, pp. 1363–1371, 2007.

[39] S. A. Fathy, M. R. Mohamed, M. A. M. Ali, A. E. el-Helaly, and A. T. Alattar, "Influence of IL-6, IL-10, IFN-γ and TNF-α genetic variants on susceptibility to diabetic kidney disease in type 2 diabetes mellitus patients," *Biomarkers*, vol. 24, no. 1, pp. 43–55, 2019.

[40] Y. Xie, T. Song, M. Huo et al., "Fasudil alleviates hepatic fibrosis in type 1 diabetic rats: involvement of the inflammation and RhoA/ROCK pathway," *European Review for Medical and Pharmacological Sciences*, vol. 22, no. 17, pp. 5665–5677, 2018.

[41] Y. W. Chen, P. L. Hsieh, Y. C. Chen, C. H. Hung, and J. T. Cheng, "Physical exercise induces excess hsp72 expression and delays the development of hyperalgesia and allodynia in painful diabetic neuropathy rats," *Anesthesia and Analgesia*, vol. 116, no. 2, pp. 482–490, 2013.

[42] L. Pawig, C. Klasen, C. Weber, J. Bernhagen, and H. Noels, "Diversity and inter-connections in the CXCR4 chemokine receptor/ligand family: molecular perspectives," *Frontiers in Immunology*, vol. 6, 2015.

[43] X. Luo, W. L. Tai, L. Sun et al., "Crosstalk between astrocytic CXCL12 and microglial CXCR4 contributes to the development of neuropathic pain," *Molecular Pain*, vol. 12, p. 174480691663638, 2016.

[44] F. Yang, W. J. Luo, W. Sun et al., "SDF1-CXCR4 signaling maintains central post-stroke pain through mediation of glial-neuronal interactions," *Frontiers in Molecular Neuroscience*, vol. 10, p. 226, 2017.

[45] H.-Y. Zhu, X. Liu, X. Miao, S. W. Di Li, and G.-Y. Xu, "Up-regulation of CXCR4 expression contributes to persistent abdominal pain in rats with chronic pancreatitis," *Molecular Pain*, vol. 13, article 1744806917697979, 2017.

[46] W. Shen, X. M. Hu, Y. N. Liu et al., "CXCL12 in astrocytes contributes to bone cancer pain through CXCR4-mediated neuronal sensitization and glial activation in rat spinal cord," *Journal of Neuroinflammation*, vol. 11, no. 1, p. 75, 2014.

[47] N. D. Jayaraj, B. J. Bhattacharyya, A. A. Belmadani et al., "Reducing CXCR4-mediated nociceptor hyperexcitability reverses painful diabetic neuropathy," *The Journal of Clinical Investigation*, vol. 128, no. 6, pp. 2205–2225, 2018.

[48] N. Kawaguchi, T. T. Zhang, and T. Nakanishi, "Involvement of CXCR4 in normal and abnormal development," *Cell*, vol. 8, no. 2, 2019.

[49] T. Nagasawa, S. Hirota, K. Tachibana et al., "Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1," *Nature*, vol. 382, no. 6592, pp. 635–638, 1996.

[50] J. Wang, W. Zhang, G.-H. He, B. Wu, and S. Chen, "Transfection with CXCR4 potentiates homing of mesenchymal stem cells in vitro and therapy of diabetic retinopathy in vivo," *International Journal of Ophthalmology*, vol. 11, no. 5, pp. 766–772, 2018.

[51] K. J. Jones, M. A. Chetram, D. A. Bethea, L. K. Bryant, V. Odero-Marah, and C. V. Hinton, "Cysteine (C)-X-C receptor 4 regulates NADPH oxidase-2 during oxidative stress in prostate cancer cells," *Cancer Microenviron*, vol. 6, no. 3, pp. 277–288, 2013.

[52] H. Huang, M. Chen, F. Liu et al., "N-Acetylcysteine tiherapeutically protects against pulmonary fibrosis in a mouse model of silicosis," *Bioscience Reports*, vol. 39, no. 7, 2019.

[53] K. N. Amin, D. Umapathy, A. Anandharaj et al., "miR-23c regulates wound healing by targeting stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) among patients with diabetic foot ulcer," *Microvascular Research*, vol. 127, 2020.

[54] Q. Li, F. I. Tian, W. L. Zhang, H. F. Ma, and S. Guan, "Effects of Xiaodu Yuji paste on protein expressions of VEGF/SDF-1 a/CXCR4 in granulation tissue of diabetic foot patients,"

Zhongguo Zhong Xi Yi Jie He Za Zhi, vol. 37, no. 2, pp. 165–168, 2017.

[55] P. Singh, M. A. Kacena, C. M. Orschell, and L. M. Pelus, "Aging-related reduced expression of CXCR4 on bone marrow mesenchymal stromal cells contributes to hematopoietic stem and progenitor cell defects," *Stem Cell Reviews and Reports*, vol. 16, no. 4, pp. 684–692, 2020.