Celecoxib ameliorates diabetic neuropathy by decreasing apoptosis and oxidative stress in dorsal root ganglion neurons via the miR-155/COX-2 axis

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Abstract. Celecoxib (CXB) is the only clinical cyclooxygenase-2 (COX-2) inhibitor. Oral administration of CXB in experimental diabetic mice effectively relieved the symptoms of diabetic neuropathy (DN); however, the molecular mechanism remains unclear. The present study aimed to investigate the potential molecular mechanisms of CXB in the treatment of DN. An *in vitro* cellular model of DN was produced by stimulating dorsal root ganglion (DRG) neurons with high glucose. Cell viability and apoptosis were assessed by Cell Counting Kit-8 assays and flow cytometry, respectively. Reactive oxygen species (ROS) kits, ELISA kits and western blotting were used to determine oxidative cellular damage. The expression level of microRNA (miR)-155 was analyzed by reverse transcription-quantitative PCR. The starBase database and dual-luciferase assays were performed to predict and determine the interaction between miR-155 and COX-2. Protein expression of neurotrophic factors, oxidative stress-related proteins and COX-2 were analyzed by western blotting. Incubation with high glucose led to a decrease in DRG neuron cell viability, facilitated apoptosis, downregulated NGF and BDNF expression, increased ROS and MDA generation and decreased SOD activity. Treatment with CXB significantly protected DRG neurons against high glucose-evoked damage. CXB promoted the expression of miR-155 and COX-2 was revealed to be a direct target of miR-155. Inhibition of COX-2 enhanced the protective effect of CXB on DRG neurons and that treatment with an miR-155 inhibitor partially rescued this effect. The present study demonstrated the involvement of the miR-155/COX-2 axis in the protective effect of CXB against high glucose-induced DN.

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Introduction

In 2019, an estimated 463 million people worldwide were affected by diabetes mellitus and 5 million people have succumbed to diabetes mellitus (1). The number of individuals with diabetes aged 20-79 years is predicted to rise to 642 million by 2040 (2,3). This increase will have adverse social and financial implications and adversely affect health systems. The global healthcare expenditure on people with diabetes was estimated to be 850 billion US dollars in 2017 (2). Diabetes results in end-organ damage and the microvascular complications of nephropathy, retinopathy and neuropathy (4). Diabetic neuropathy (DN), one of the most common complications of diabetes, affects ≥50% of patients with diabetes (5). Its most prominent feature is nerve fiber damage and/or dysfunction, and symptoms of DN usually include numbness, tingling, pain and weakness (6). Although there are numerous pharmacological methods to treat DN, they lack efficacy and cause adverse reactions, such as neuropsychiatric nature, peripheral edema and weight gain (7). In addition to strict glycemic control, researchers have sought potential therapeutic targets and novel pharmacological alternatives (8).

A previous study revealed increased oxidative stress in the pathogenesis of experimental DN. Elevated extra- and intracellular glucose concentrations result in the impairment of antioxidant defense in patients with diabetes and in diabetic animal models (9,10). Hyperglycemia has been proposed to promote reactive oxygen species (ROS) and malondialdehyde (MDA) accumulation and reduce the activity of superoxide dismutase (SOD) (11). An increase in ROS and MDA is directly neurotoxic, promotes neuronal apoptosis (12) and may inhibit mitochondrial respiratory enzymes, resulting in insufficient neural energy production and insufficient neurological function (13). Cyclooxygenase-2 (COX-2) mediates the development of inflammation and is involved in oxidative stress and ROS production (14). Short-term selective chemical COX-2 inhibition in rats and COX-2 gene inactivation in

mice prevented functional and biochemical peripheral nerve defects caused by diabetes (13,15). Furthermore, nuclear factor erythroid-2-related factor 2 (Nrf2) is involved in antioxidant stress (16). Numerous studies have indicated that persistent hyperglycemia decreases Nrf2 expression (17-19). This down-regulation of Nrf2 causes various microvascular changes, ultimately leading to DN (18). Moreover, COX-2-dependent electrophilic oxygen molecules have been demonstrated to act as anti-inflammatory agents by their activation of Nrf2-dependent antioxidant response elements (20). Therefore, selective COX-2 inhibition may be useful for preventing or delaying DN (21).

Celecoxib (CXB) is a nonsteroidal anti-inflammatory drug widely used clinically to treat pain and inflammation (22,23). It has been hypothesized that nonsteroidal anti-inflammatory drugs relieve pain primarily by suppressing the activity of COX proteins (COX-1, COX-2 and COX-3) (24). Among them, COX-2 has been described as a target of CXB (25) and that CXB is the only COX-2 inhibitor currently in clinical use (26). Oral administration of CXB in experimental diabetic mice effectively relieved neuropathic DN pain (27,28); however, the molecular mechanism remains unclear.

MicroRNAs (miRNAs or miRs) are endogenous noncoding single-stranded RNAs consisting of ~22 nucleotides (29). The majority of miRNAs are involved in regulating post-transcriptional gene expression (30). It has been hypothesized that miRNAs regulate 1/3 of human genes (31). Furthermore, miRNAs serve a key role in numerous biological processes. Apoptosis, differentiation and metabolism are regulated by base-pairing with targets, causing target mRNA degradation or inhibiting translation (32). The regulation of miR-155 in acute and chronic inflammatory responses has been extensively studied (33,34). Moreover, miR-155 has been demonstrated to serve an important role in peripheral DN (35). miR-155 protects dorsal root ganglion (DRG) neurons by downregulating the inflammatory response (36). However, it remains unclear whether miR-155 affects oxidative stress in DN.

The present study aimed to investigate the molecular mechanism by which CXB relieves DN using a cellular model of DN generated by stimulating DRG neurons with hyperglycemic conditions. Furthermore, the present study also researched the role of CXB in regulating the expression of miR-155 and COX-2, thereby providing a possible explanation for the effect of CXB on DN.

Materials and methods

Cell culture. Mouse DRG neurons were purchased from Qincheng Biotechnology Co., Ltd. (cat. no. MIC-QC-242; Beijing Solarbio Science & Technology Co., Ltd.). DRG neurons were cultured in DMEM with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin (all Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C with 5% CO_2 in a humid atmosphere. The neurons were cultured for a few passages prior to subsequent experiments.

Establishment of the cell damage model and CXB treatment. To mimic diabetes and DN *in vitro*, DRG neurons were cultured in complete medium supplemented with 50 mM glucose (cat. no. G8150; Beijing Solarbio Science & Technology Co., Ltd.). DRG neurons were maintained under high-glucose conditions at 37˚C with 5% CO_2 for 48 h (D) or 72 h (DN). DRG neurons in the negative control (NC) group were cultured in complete medium containing 25 mM glucose conditions at 37˚C with 5% CO_2 for 48 h, which is optimal for DRG neuron survival and growth (37).

To determine the effect of CXB (cat. no. IC0230; Beijing Solarbio Science & Technology Co., Ltd.) on DN, DRG neurons in the DN group were incubated with CXB at various concentrations (0, 1, 5, 15, 30 or 50 μM) for 24 h prior to subsequent experiments.

Cell transfection. miR-155 inhibitors (5'-ACCCCUAUCACA AUUAGCAUUAA-3'), NC inhibitors (5'-CAGUACUUUUGU GUAGUACAA-3'), miR-155 mimics (5'-UUAAUGCUAAUC GUGAUAGGGGU-3'), mimic NC (5'-UCACAACCUCCU AGAAAGAGUAGA-3') and predesigned vectors expressing siRNAs targeting COX-2 mRNAs (si-COX-2, 5'-AATGTC CGGGTACAATCGCACCCTGTCTC-3') were purchased from Cytiva. Scrambled siRNA (si-control, 5'-AATTCT CCGAACGTGTCACGT-3'; Qiagen GmbH) was used as the NC for si-COX-2. A total of $2x10^5$ DRG neurons of the DN group were cultured in 24-well plates and transfected using Lipofectamine™ 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-155 inhibitors, miR-155 mimics, NC inhibitors and mimic NC were used at a concentration of 100 nM and si-COX-2 and si-controls were used at a concentration of 50 nM. Cells were treated with CXB at 48 h post-transfection, after which cell suspensions were collected for further analysis.

Cell viability. Cell viability was measured using a Cell Counting Kit-8 (CCK-8; cat. no. C0038; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. DRG neurons were cultured into a 96-well microplate ($5x10^3$ cells/well) and treated with glucose, CXB and/or transfected. A total of 10 μl of CCK-8 reagent was added to the microplates and incubated at 37˚C for 2 h. Absorbance at 450 nm (A450) was detected using a microplate reader (Bio-Rad Laboratories, Inc.). Cell growth curves were plotted based on the average A450 value from 3 replicate measurements.

Apoptosis analysis. Apoptosis was determined with an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (cat. no. E606336-0500; Sangon Biotech, Co. Ltd.) according to the manufacturer's protocol. DRG neurons under various treatment conditions were seeded into 6-well plates ($5x10^5$ cells/well) and reacted with 5 μl of Annexin V-FITC and 10 μl of PI in the dark at a room temperature for 5 min. Flow cytometric analysis was performed using a flow cytometer (FACSCalibur; BD Biosciences) and FlowJo software version 7.6 (FlowJo, LLC). The results reflect the combination of early and late apoptotic cells.

ROS assay. ROS generation was detected using a ROS assay kit (cat. no. S0033S; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. DRG neurons under various treatment conditions were seeded into 6-well plates ($5x10^5$ cells/well). Then, 10 μM 2,7-dichlorodi-hydrofluorescein

diacetate (DCFH-DA) was added into each well and incubated at 37˚C for 30 min in the dark. The fluorescence intensity at 485 and 530 nm was examined with a flow cytometer (FACSCalibur; BD Biosciences) to evaluate ROS generation.

ELISA. ELISAs were performed with ELISA kits for malondialdehyde (MDA; cat. no. SBJ-M0411; SenBeiJia Biological Technology Co., Ltd.) and superoxide dismutase (SOD; cat. no. ml037856; Mlbio). A total of $1x10^5$ cells under various treatment conditions (glucose, CXB and/or transfection) added to 96-well microplates and processed according to the manufacturer's protocol. Conditioned mediums were collected from the wells at 24 h after processing. Finally, the absorbance at A450 was detected with a microplate reader (Bio-Rad Laboratories, Inc.) and quantities were calculated with standard curves.

Reverse transcription-quantitative PCR (RT-qPCR). A total of $5x10^6$ DRG neurons/sample were harvested following treatments or transfections. Total cellular RNA was isolated using TRIzol™ Reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). miR-155 expression was examined by TransScript® Green miRNA Two-Step RT-qPCR SuperMix (cat. no. AQ202-01; Beijing Transgen Biotech Co., Ltd.). The specific primers used were as follows: miR-155 forward, 5'-CTGTATCAA AAGGCCAACTGAA-3' and reverse, 5'-GTGTCTATCCTT ATGAATCGCCA-3'; U6 forward, 5'-AACGAGACGACG ACAGAC-3' and reverse, 5'-GCAAATTCGTGAAGCGTT CCATA-3'. PCR amplification was then conducted using a LightCycler 480 instrument (Roche Diagnostics). The thermocycling conditions were as follows: 95˚C for 10 sec followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. U6 was used as internal controls. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (38).

Western blotting. DRG neurons under various treatment conditions were seeded into 6-well plates ($5x10^5$ cells/well). Crude cell lysates were harvested using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's protocol. The purity of the protein in extracts was examined by the BCA method. Proteins (100 μg/lane) were separated by 10% SDS-PAGE and transferred to a PVDF membranes. Nonspecific protein binding was prevented with the addition of blocking buffer [5% milk, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20] for 40 min at a room temperature. The membranes were incubated with specific primary antibodies (1:2,000; Abcam) against nerve growth factor (NGF; cat. no. ab52918), brain-derived neurotrophic factor (BDNF; cat. no. ab226843), COX-2 (cat. no. ab188183), kelch-like ECH-associated protein 1 (Keap1; cat. no. ab227828), nuclear factor erythroid-2-related factor 2 (Nrf2; cat. no. ab137550), heme oxygenase-1 (HO-1; cat. no. ab189491), superoxide dismutase 1 (SOD1; cat. no. ab13498), SOD2 (cat. no. ab137037) or β-actin (cat. no. ab115777) were incubated in blocking buffer at 4˚C overnight. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin F (1:10,000; cat. no. ab205718; Abcam) for 60 min in the dark (at room temperature), and developed using an ECL reagent (cat. no. 32209; Thermo Fisher Scientific, Inc.). Membranes were exposed using chemiluminescence apparatus (Bio-Rad Laboratories, Inc.). ImageJ software (version 1.8.0; National Institutes of Health) was used to quantify the protein grayscale.

starBase database analysis. starBase database (http://starbase.sysu.edu.cn) provides a widely-used noncoding RNA interaction from crosslinking-immunoprecipitation and high-throughput sequencing (CLIP-seq) (39). The database was used to predict potential target sequences of miR-155 and COX-2.

Dual-luciferase reporter assay. The direct binding of COX-2 and miR-155 was verified by dual-luciferase reporter assays. Portions of the wild-type (WT) and mutant (MUT) 3'-untranslated regions (UTRs) of COX-2 mRNA containing the predicted miR-155-targeting regions were synthesized and inserted into the pGL3-report luciferase reporter vector (Sigma-Aldrich; Merck KGaA). miR-155 mimics and NC mimics were cotransfected into cells with pGL3-3'-UTR wild-type or mutant plasmid DNA using Lipofectamine® 3000 (cat. no. L3000008; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 24 h, relative luciferase activity was analyzed using a dual-luciferase reporter gene analysis system (Promega Corporation) and *Renilla* luciferase reference plasmids were used for standardization.

Statistical analysis. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp.). Unpaired Student's t-test was used to evaluate the differences between two groups. One-way ANOVA with Tukey's Multiple Range post hoc test was used to determine differences between multiple groups. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DRG neurons are injured by high glucose. To detect the effect of glucose on DRG neurons, DRG neurons were treated with glucose for 48 or 72 h. Cell viability was suppressed and apoptosis was induced by 50 mM glucose for 48 h compared with the NC group and cell survival was further inhibited by 50 mM glucose treatment for 72 h ($P<0.05$; Fig. 1A and B). These results indicated that prolonged exposure to a high glucose concentration damaged the DRG neurons. Thus, high-glucose treatment of DRG neurons for 48 or 72 h mimicked D or DN, respectively, *in vitro*.

CXB alleviates the inhibitory effect of DN on DRG neuron survival. CXB (various doses, 1-50 μM) was utilized to treat DRG neurons. Cell viability of DRG neurons was examined to evaluate the cytotoxicity of CXB, which indicated that cell viability was unchanged by treatment with CXB at different concentrations (Fig. 2A). To examine the effect of CXB on DRG neurons, DRG neurons were treated with CXB at various concentrations. CXB increased DRG neuron viability in the DN group to a certain extent ($P<0.05$; Fig. 2B). Considering that DN DRG neurons treated with 30 μM CXB exhibited the highest

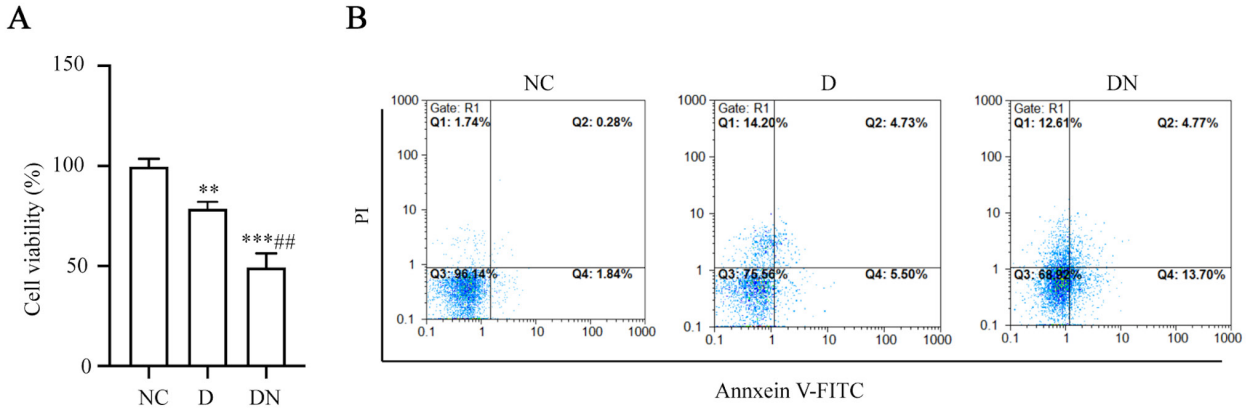

Figure 1. Dorsal root ganglion neurons are injured by high glucose. Following normal glucose treatment for 48 h (NC) or high glucose treatment for 48 h (D) and 72 h (DN), (A) cell viability was detected using the Cell Counting Kit-8 assay. (B) Cell apoptosis rates were measured using flow cytometry. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001 vs. NC group, and $^{\#\#}$P<0.01 vs. D group using One-way ANOVA with Tukey's post hoc test. NC, negative control; D, diabetes; DN, diabetic neuropathy; PI, propidium iodide.

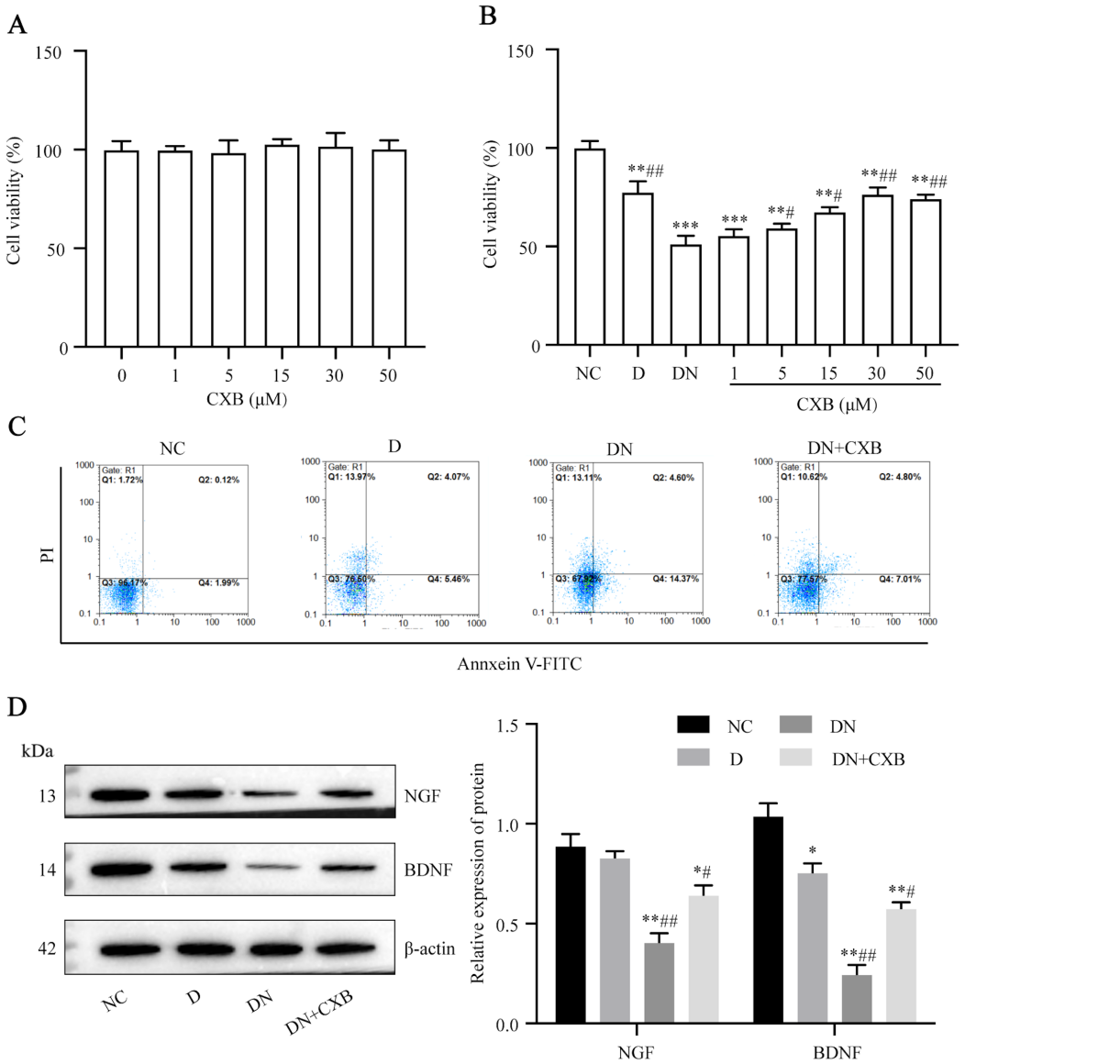

Figure 2. CXB alleviates the inhibitory effect of DN on dorsal root ganglion neuron survival. Following treatment on NC neurons with 0-50 μM CXB, (A) the cytotoxicity of CXB was measured using the CCK-8 assay. Following treatment of DN neurons with 0-50 μM CXB, (B) cell viability and (C) cell apoptosis rates were measured using the CCK-8 assay and flow cytometry, respectively. (D) Protein levels of NGF and BDNF were measured using western blotting. Data are presented as the mean ± SD. In (B), **P<0.01 and ***P<0.001 vs. NC group, and $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. DN group using One-way ANOVA with Tukey's post hoc test. In (D), *P<0.05 and **P<0.01 vs. NC group, and $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. D group, using One-way ANOVA with Tukey's post hoc test. CXB, celecoxib; DN, diabetic neuropathy; CCK-8, Cell Counting Kit-8; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; PI, propidium iodide.

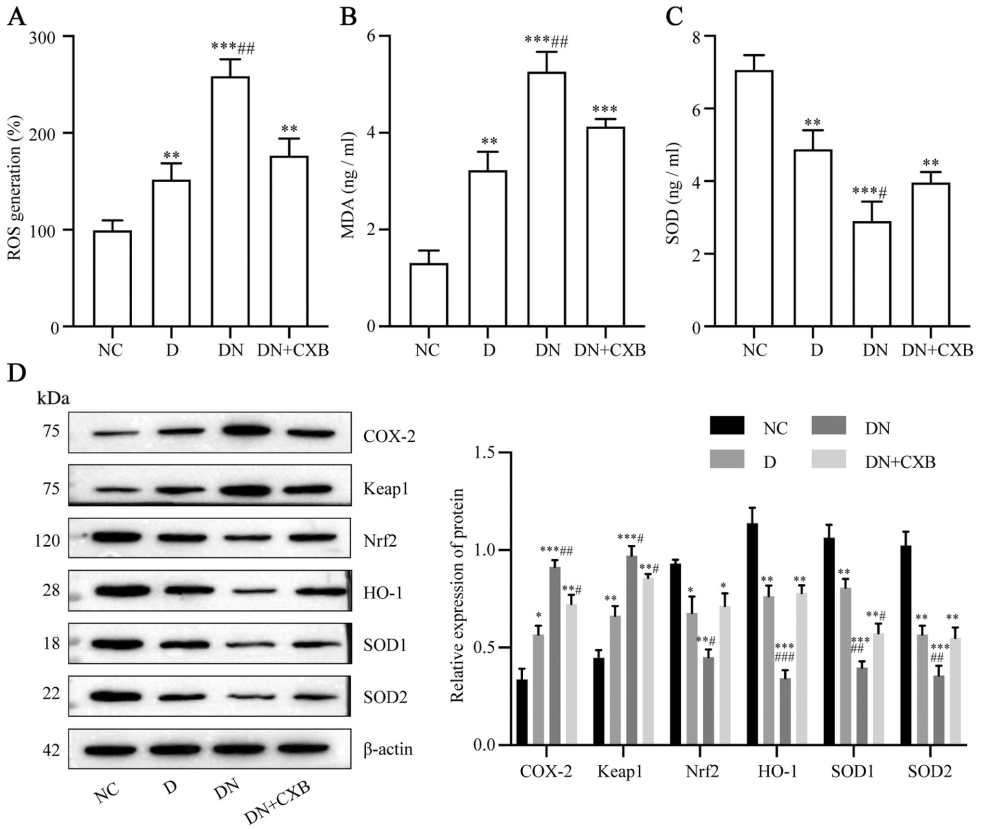

Figure 3. CXB ameliorates DN-induced oxidative stress in dorsal root ganglion neurons. (A) ROS generation was measured using an ROS assay kit. (B) MDA and (C) SOD levels were measured using ELISA kits. (D) The levels of oxidative stress-related proteins were measured using western blotting. Values are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. NC group, and $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. D group, using One-way ANOVA with Tukey's post hoc test. CXB, celecoxib; DN, diabetic neuropathy; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; NC, negative control; D, diabetes; COX-2, cyclooxygenase-2; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2.

viability, 30 μM was used as the optimum CXB dose in subsequence experiments. Additionally, apoptosis in DN neurons was attenuated by CXB treatment (Fig. 2C). Furthermore, DN-induced neurotrophic factors NGF and BDNF suppression was attenuated with CXB treatment (Fig. 2D; P<0.05). These results indicated that CXB rescued DN-mediated inhibition of DRG neuron survival to a certain extent.

CXB ameliorates DN-induced oxidative stress in DRG neurons. DRG neurons were treated with 30 μM CXB and the protective effect of CXB against oxidative stress in neurons was evaluated. The ROS and MDA levels in the DN group were decreased by CXB treatment (P<0.05; Fig. 3A and B). However, the SOD levels were enhanced by CXB treatment (P<0.05; 3C). Additionally, the levels of oxidative stress-related proteins in each group were assessed. The levels of COX-2 and Keap1 were reduced by CXB treatment, while the levels of Nrf2, HO-1, SOD1 and SOD2 were partially increased by CXB treatment (P<0.05; Fig. 3D). These data indicated that CXB effectively ameliorated oxidative stress in DRG neurons in the DN group.

CXB prevents DN-induced injury in DRG neurons by upregulating miR-155. The regulatory effect of miR-155 on CXB-alleviated DN was investigated. RT-qPCR data demonstrated that miR-155 expression was suppressed in the DN group (Fig. 4A). Furthermore, miR-155 expression was increased in a dose-dependent manner by CXB treatment (P<0.05). miR-155 expression in DN-treated DRG neurons was decreased by miR-155 inhibitor transfection (P<0.05; Fig. 4B). Following this, the importance of miR-155 in the protective function of CXB was determined. Compared to transfection with NC inhibitor, transfection with miR-155 inhibitor significantly decreased cell viability (Fig. 4C), promoted apoptosis (Fig. 4D), suppressed NGF and BDNF expression (Fig. 4E) and accelerated oxidative damage (Fig. 4F-I; all, P<0.05). Moreover, the protective functions of CXB against DN-induced damage in DRG neurons were partially abrogated by inhibition of miR-155 expression (Fig. 4C-I). These results indicated that miR-155 served an important role in CXB-mediated alleviation of DN.

Prediction of COX-2 as a target of miR-155. The online bioinformatics tool starBase predicted COX-2 as a target of miR-155. The complementary binding sites of COX-2 and miRNA-155 are presented in Fig. 5A. To further confirm the interaction between miR-155 and COX-2, luciferase reporter gene vectors that expressed WT or MUT 3'UTR of the downstream target luciferase gene COX-2 were constructed. The results demonstrated a significant decrease in luciferase activity in the COX-2 3'UTR-WT/miR-155 mimic group compared with the COX-2 3'UTR-MUT/miR-155 mimic group

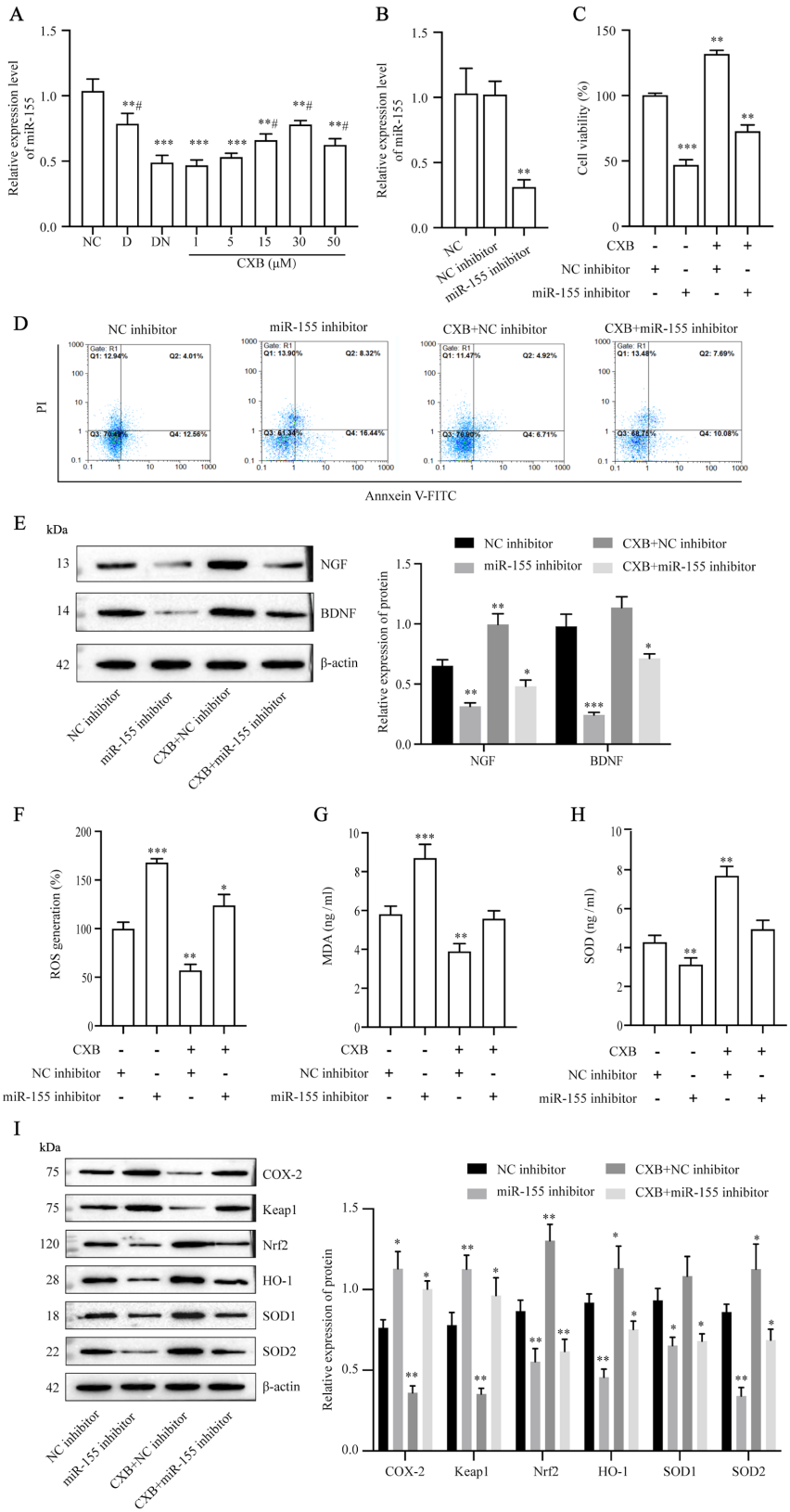

Figure 4. CXB prevents DN-induced injury in dorsal root ganglion neurons by upregulating miR-155. Following treatment of DN neurons with 0-50 μM CXB, (A) the expression of miR-155 was measured using RT-qPCR. (B) Expression of miR-155 in DN neurons following miR-155 inhibitor transfection was measured using RT-qPCR. Following treatment with 30 μM CXB and/or miR-155 inhibitor or NC inhibitor transfection, (C) cell viability, (D) the cell apoptosis rate and (E) protein levels of neurotrophic factors were detected using the Cell Counting Kit-8 assay, flow cytometry and western blotting, respectively. (F) ROS generation was measured using an ROS assay kit, (G) MDA and (H) SOD were measured using ELISA kits and (I) the levels of oxidative stress-related proteins were measured using western blotting. Data are presented as the mean ± SD. In (A), **P<0.01 and ***P<0.001 vs. NC group, and $^{\#}$P<0.05 vs. DN group using One-way ANOVA with Tukey's post hoc test. In Fig. 4B-I, *P<0.05, **P<0.01 and ***P<0.001 vs. NC inhibitor group using One-way ANOVA with Tukey's post hoc test. CXB, celecoxib; DN, diabetic neuropathy; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; D, diabetes; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase-2; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; PI, propidium iodide.

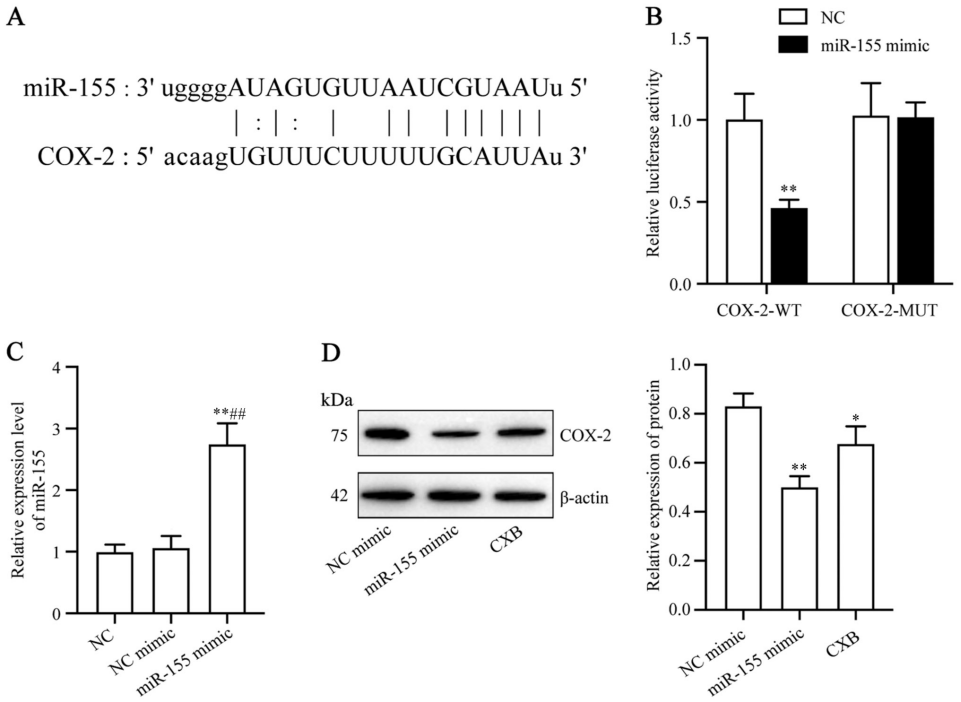

Figure 5. Prediction of COX-2 as a target of miR-155. (A) The starBase database predicted complementary binding sites in COX-2 and miR-155. (B) The targeted relationship between COX-2 and miR-155 was identified using a dual-luciferase reporter gene assay. (C) miR-155 expression in diabetic neuropathy neurons following miR-155 mimic transfection was measured using reverse transcription-quantitative PCR. (D) Protein levels of COX-2 in dorsal root ganglion neurons was assessed using western blotting. Data are presented as the mean ± SD. In (B), **P<0.01 compared with the COX-2-MUT group using unpaired student's t-test. In (C), **P<0.01 vs. NC group and $^{\#\#}$P<0.01 vs. NC mimic group using One-way ANOVA with Tukey's post hoc test. In (D), *P<0.05 and **P<0.01 vs. NC mimic group using One-way ANOVA with Tukey's post hoc test. COX-2, cyclooxygenase-2; miR, microRNA; NC, negative control; WT, wild type; MUT, mutant; CXB, celecoxib.

(P<0.01; Fig. 5B). miR-155 mimic transfection efficiency was detected using RT-qPCR (P<0.05; Fig. 5C). The regulation of COX-2 by miR-155 in DRG neurons was then investigated. The protein expression level of COX-2 was significantly decreased after cells were treated with miR-155 mimics compared with NC mimics (P<0.05; Fig. 5D). These results demonstrated that miR-155 targeted COX-2.

CXB prevents DN-induced injury to DRG neurons by regulating the miR-155/COX-2 axis. The mechanism by which miR-155 and COX-2 are involved in CXB-mediated alleviation of DN was investigated. Vectors that expressed si-COX-2 or si-COX-2 plus miR-155 inhibitor were transfected into DRG neurons in the DN group. The protein expression of COX-2 was decreased by si-COX-2 transfection (P<0.05; Fig. 6A). Following this, the association between miR-155 and COX-2 with CXB-mediated alleviation of DN was investigated. Compared to the cell viability in the DN group and CXB treatment group, transfection with si-COX-2 plus CXB treatment increased cell viability (P<0.05; Fig. 6B), decreased apoptosis (Fig. 6C), upregulated NGF and BDNF expression (P<0.05; Fig. 6D) and reduced oxidative damage (P<0.05; Fig. 6E-H). Furthermore, the protective functions of CXB against DN-induced damage in DRG neurons were partially reduced by si-COX-2 and miR-155 inhibitor transfection plus CXB treatment (P<0.05; Fig. 6B-H). In summary, these results indicated that CXB attenuated DN-induced DRG neuron damage and oxidative stress by regulating the miR-155/COX-2 axis.

Discussion

In the present study, DRG neurons were stimulated with high glucose to mimic DN *in vitro* and the therapeutic effect of CXB on DN was evaluated. High-glucose conditions led to reduced cell viability and increased apoptosis and ROS generation, indicating that DRG neurons were damaged. Additionally, the function of DRG neurons may have been disturbed, as evidenced by the decreased expression of NGF and BDNF. CXB treatment significantly protected DRG neurons against DN-induced damage. The neuroprotective properties of CXB may be due to the upregulated expression of miR-155, which was inversely associated with expression of COX-2. These *in vitro* data provided evidence that CXB attenuated DN-induced damage and oxidative stress in DRG neurons by regulating the miR-155/COX-2 axis.

During the progression of diabetes, long-term hyperglycemic exposure induces neuronal apoptosis, which leads to nerve dysfunction and, ultimately, DN (40). Hyperglycemic conditions increase oxidative stress. Hyperglycemia induces free radicals and produces ROS and MDA, which reduces protein and unsaturated fatty acids synthesis (11,41,42). Furthermore, hyperglycemia impairs the endogenous antioxidant defense system in patients with diabetes (9). SOD is the most potent antioxidant *in vivo* (43). SOD catalyzes superoxide into oxygen and hydrogen peroxide (44). However, persistent hyperglycemic stimulation decreases SOD activity, which subsequently aggravates damage to DRG neurons by oxidative stress (45,46). Consistently, the results of the present study

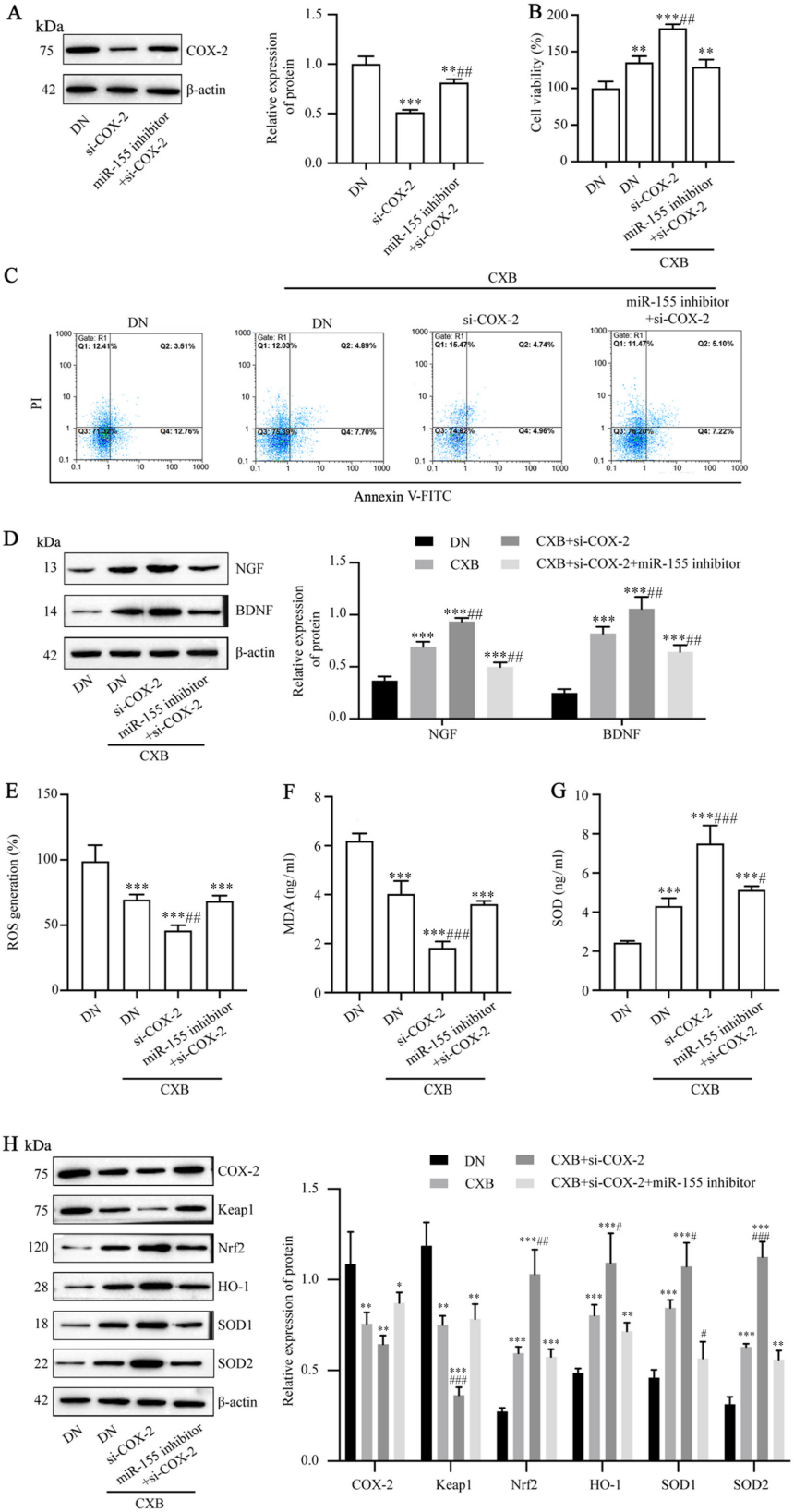

Figure 6. CXB prevents DN-induced injury in dorsal root ganglion neurons by regulating the miR-155/COX-2 axis. (A) The protein levels of COX-2 following transfection with si-COX-2 or si-COX-2 plus miR-155 inhibitor were assessed using western blotting. Following treatment with 30 μM CXB and transfection with si-COX-2 or si-COX-2 plus miR-155 inhibitor, (B) cell viability, (C) cell apoptosis rate and (D) protein levels of neurotrophic factors were detected using a Cell Counting Kit-8 assay, flow cytometry and western blotting, respectively. (E) ROS generation was measured using an ROS assay kit, (F) MDA and (G) SOD were measured using ELISA kits and (H) the levels of oxidative stress-related proteins were measured using western blotting. In (A), **P<0.01 and ***P<0.001 vs. DN group, and $^{\#\#}$P<0.01 vs. si-COX-2 group using One-way ANOVA with Tukey's post hoc test. In (B-H), *P<0.05, **P<0.01 and ***P<0.001 vs. DN group, and $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. DN+CXB group using One-way ANOVA with Tukey's post hoc test. CXB, celecoxib; DN, diabetic neuropathy; miR, microRNA; COX-2, cyclooxygenase-2; si-COX, siRNAs targeting COX-2 mRNAs; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; PI, propidium iodide; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2.

indicated that the exposure of DRG neurons to high glucose led to an increase in ROS and MDA and a decrease in SOD activity. CXB, the only clinically used COX-2 inhibitor, is involved in regulating apoptosis and ROS production in a variety of cells, such as myocardia cells and neurons (47-49). The present study showed that CXB has neuroprotective functions in a DN cell model. As revealed by *in vitro* experiments, CXB protected DRG neurons from the high glucose-induced loss of viability, apoptosis and ROS production. DRG neurons are the primary neurons of the sensory pathway (50). The unique structure of the stem axons of DRG neurons, which bifurcate into peripheral and central axon branches, may have important implications for their function in health and disease (51). Additionally, NGF and BDNF serve crucial roles in the survival of human DRG neurons (52). CXB was revealed to reduce memory impairment by regulating the BDNF-tropomyosin receptor kinase B signaling pathway in diabetic rat models (53). The data in the present study demonstrated that CXB increased BDNF and NGF expression in DRG neurons, thereby increasing DRG neuron survival.

Glucose-mediated oxidative stress and alterations in COX pathway activities have been implicated in the pathogenesis of experimental DN (15,54). Several DN treatments targeting COX-2 have been reported, including rutin in combination with nimesulide (55), *Juglans regia* L. leaf extract (56) and curcumin (57). As a known inhibitor of COX-2, CXB has been demonstrated to reduce neuropathic pain caused by DN in rats (27,58). However, to the best of our knowledge, the present study is the first to reveal a possible mechanism for CXB in the treatment of DN. COX-2, also known as prostaglandin H synthase 2, is a key enzyme that oxidizes arachidonic acid (59) and the products of COX-2 activity participate in various physiological and pathophysiological processes, including pain (60), inflammation, oxidative stress (61) and cancer (62). Groeger *et al* (63) demonstrated that COX-2 activates Nrf2 expression by forming a heterodimer with a small Maf protein and binding to the ARE upstream promoter region and that COX-2 regulates the expression of various antioxidant and anti-inflammatory genes, such as Nrf2 and HO-1. The Nrf2/HO-1 signaling pathway is involved in the oxidative stress response (64). In the present study, CXB impacted the levels of Nrf2 and HO-1 by regulating the expression of COX-2. These results indicated that COX-2-mediated oxidative stress may play a role through the Nrf2/HO-1 signaling pathway.

As CXB is a drug for pain and inflammation (22,23), the underlying mechanisms of the function of CXB have been preliminarily investigated. Several studies have indicated that CXB exerts its functions by regulating miRNA expression patterns. For example, CXB inhibited breast cancer by increasing miR-222 (65) and miRNA-145 was reported to be involved in CXB-mediated inhibition of the epithelial-to-mesenchymal transition in bladder cancer (66). The results of the present study demonstrated that miR-155 is a target of CXB, as its expression was positively regulated by CXB. miR-155 has been widely studied for its reported functions in proliferation (67), apoptosis (68) and lipid metabolism (69). In DN, miR-155 was revealed to protect DRG neurons by downregulating the expression of tumour necrosis factor-associated factor 2, Notch receptor 2 and sortillin 1 (36). The present study demonstrated that miR-155 was involved in regulating oxidative stress by targeting COX-2. Furthermore, miR-155 may be a key molecular target for the diagnosis and treatment of DN.

This study preliminarily demonstrated the potential therapeutic effect of CXB in DN cell model. In order to better clarify the therapeutic effect of CXB on DN, the protective effect of CXB on DN *in vivo* will be further explored, and the underlying molecular mechanisms investigated. In conclusion, the results of the present study may be beneficial for research in related fields for the development of novel treatment strategies for DN.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

WR contributed substantially to the study conception and drafting the manuscript. XC and LZ were involved in the study conception and design. XC, LZ, TK and XW performed literature research and the experimental studies. XC, LZ and LC acquired data and edited the manuscript. SL and JH performed data and statistical analyses. WR, XC and LZ were involved in critically revising the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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