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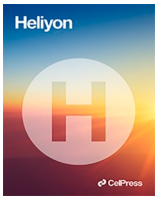

Research article

Calcitonin gene-related peptide attenuated discogenic low back pain in rats possibly via inhibiting microglia activation

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ABSTRACT

Discogenic low back pain (DLBP) is a multifactorial disease and associated with intervertebral disc degeneration. Calcitonin gene-related protein (CGRP) plays a critical role in pain processing, while the role in DLBP remains unclear. This study aims to investigate the anti-nociceptive role and related mechanisms of CGRP in DLBP. Here we established the DLBP rat and validated the model using histology and radiography. Minocycline, a microglial inhibitor, and CGRP were intrathecally injected and the behavioral test was performed to determine hyperalgesia. Further, BV2 microglial cells and microglial activation agent lipopolysaccharide (LPS) were employed for the in vitro experiment. We observed obvious lumbar intervertebral disc degeneration and hyperalgesia at 12 weeks postoperation in DLBP group, with significantly activated microglia in the spinal cord. CGRP treatment significantly inhibited the upregulation of proinflammatory cytokines and NLRP3/caspase-1 expression induced by LPS in BV2 cells, whereas treatment with CGRP alone had little effect on BV2 cells. The intrathecal injection of CGRP into DLBP rats relieved mechanical and thermal hyperalgesia, reverted the microglial activation and decreased the expression of NLRP3/caspase-1, similar to the effects produced by minocycline. Our results provide evidence that microglial activation in the spinal cord play a key role in hyperalgesia in DLBP rats. CGRP alleviates DLBP induced hyperalgesia and inhibits microglial activation in the spinal cord. Regulation of CGRP and microglial activation may provide a new strategy for ameliorating DLBP.

1. Introduction

Low back pain (LBP) is a multifactorial disease that involves physical and psychological factors and functional and structural changes in the brain. It was reported that 60%–80% of the population experiences at least one episode of LBP [1]. Intervertebral disc degeneration (IDD) is considered a significant cause of LBP [2]. Discogenic LBP (DLBP) has been used to describe LBP associated with IDD and without herniation or other anatomical deformity. To date, there is still no effective method to treat DLBP.

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Calcitonin gene-related protein (CGRP) and inflammatory cytokines in degenerative disc have been implicated as key players in DLBP [3]. However, the pain in DLBP patients who underwent total disc replacement was relieved but not eliminated [4]. Thus, it suggested that in addition to the degenerative disc, there are other factors that contribute to DLBP. It is well known that microglia play a major role in the development and maintenance of neuropathic pain, through central sensitization and long-term potentiation in the spinal nociceptive responses [5–8]. Miyagi et al. [9] observed increased microglia in the spinal dorsal horn in the LBP rats induced by disk injury. These findings suggested that microglia activation in the spinal cord is closely related to DLBP.

The neuropeptide CGRP involved in both modulation and transmission of pain is widely distributed in both peripheral nervous system and central nervous system (CNS) [10]. CGRP has been implicated in both pro- and antinociceptive effects in CNS. Zheng et al. [11] reported the changes of CGRP expression in the spinal cord from different neuropathic pain models. The expression level of CGRP in the spinal cord was up-regulated after sciatic nerve crush or sciatic nerve transection, but down-regulated in the sciatic nerve ligation rat models. And studies have shown that CGRP induced an antinociceptive effect in several brain areas [10,12], whereas the effects of CGRP in spinal cord remains uncertain.

Recently it was reported that the degenerative disc might induce the neuroinflammatory microenvironment in the spinal cord and drive activation of microglia [13]. The neuroinflammatory markers released by the activated microglia in the spinal cord, like the interleukin (IL)-1β, could in turn enhance the inflammatory milieu and have been implicated as key mediators of pain. The NOD-like receptor protein 3 (NLRP3) inflammasome has been found to trigger the activation of caspase-1 and induce the maturation of IL-1β [14], which is crucial for the regulation of microglia activation [15,16]. Several studies have suggested that the activation of NLRP3/caspase-1/IL-1β pathway in intervertebral disc was involved in the degenerative process and low back pain [17–19]. It is still unknown whether the NLRP3/caspase-1/IL-1β pathway in the spinal cord is related to the DLBP. CGRP could reduce the NLRP3 and IL-1β protein expression induced by lipopolysaccharide (LPS) in murine macrophages [20]. And Xu et al. [21] reported that CGRP alleviated the inflammatory reaction in mice keratitis and reduced the levels of NLRP3 and IL-1β in A. fumigatus stimulated-macrophages. It is noteworthy that CGRP was demonstrated to inhibit the microglia activation induced by LPS in vitro [22]. These findings prompted us to investigate whether CGRP in the spinal cord plays roles in regulating microglia activation and NLRP3/caspase-1/IL-1β pathway in DLBP models.

In this study, the DLBP rat model was established through unbalanced dynamic and static forces which induced by destroying lumbar spine stabilizers. And the intrathecal infusions of the microglial inhibitor minocycline or CGRP were applied to the DLBP rats to investigate the role of microglia and CGRP in DLBP. We found that both CGRP and minocycline alleviate hyperalgesia and inhibit microglial activation in the spinal cord in DLBP rats, suggesting a potentially therapeutic strategy for DLBP.

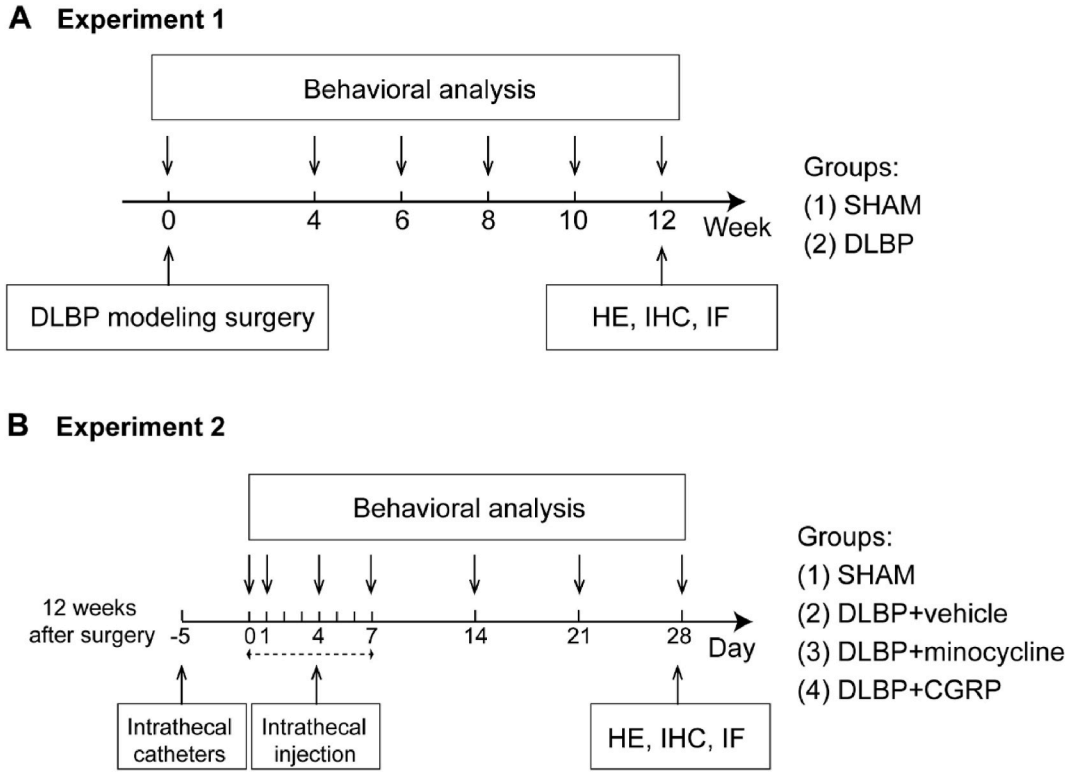

Fig. 1. Animal experimental designs and groups. A Experiment 1: Changes in pain threshold, CGRP expression and microglial activation in DLBP rats. **B** Experiment 2: The effects of CGRP and minocycline treatment on hyperalgesia, microglial activation, and the NLRP3/caspase-1 after DLBP.

2. Material and methods

2.1. In vivo experimental designs and animal groups

The surgical procedures were performed on male 8-week-old Sprague-Dawley rats (200–250 g). All rats were housed under standard conditions with a 12-h light to dark cycle and ad libitum access to food and water. Ethical approval for performing animal experiments was obtained from the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. The animal experiments were conducted in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and complied with the ARRIVE guidelines. As shown in Fig. 1A and B, there were two part of animal experiments in this study.

2.1.1. Animal experiment 1: changes in pain threshold, CGRP expression and microglial activation in DLBP rats

Twelve rats were randomly divided into two groups: a sham operation (SHAM) group and a DLBP group. Rats in the DLBP group were subjected to a surgical procedure [23] that could induce unbalanced dynamic and static forces. The paraspinal musculature and ligaments, along with part of the facet joints, were excised to destroy the static and dynamic lumbar stabilizers. For rats in the SHAM group, a posterior skin incision was made. According to previous studies [23,24], the lumbar IDD induced by unbalanced dynamic and static forces was significant in the rat from 6 weeks after surgery. Thus the assessment of the pain threshold was done 1 day before surgery (baseline) and at 4, 6, 8, 10 and 12 weeks after surgery. Subsequently, the rats were examined by X-ray radiography (KXO-32R, Toshiba, Japan) and 3.0-T MRI (Siemens, Erlangen, Germany) to assess the IDD. After the radiological examination, all animals were euthanized by an overdose pentobarbital injection. The L5/6 IVDs and the L3–L5 spinal cord segments were harvested for Western blot, immunohistochemical and immunofluorescent analysis.

2.1.2. Animal experiment 2: the effects of CGRP and minocycline treatment on hyperalgesia, microglial activation, and the NLRP3/caspase-1 after DLBP

A total of 24 rats were randomly divided into four groups: (1) SHAM group; (2) DLBP + vehicle group; (3) DLBP + minocycline group; (4) DLBP + CGRP group. The DLBP rats were intrathecally treated with drugs or an equivalent volume of saline once daily for 7 days at 12 weeks after the operation. Drugs were diluted with saline and intrathecally injected through the implanted catheter in a 10 μl volume of solution followed by 10 μl of saline for flushing. In the DLBP + vehicle group, rats were administrated intrathecally with saline. In the DLBP + minocycline group, rats were administrated intrathecally with microglia inhibitor minocycline (20 mM; S4226; Selleck Chemicals, USA). And the rats in the DLBP + CGRP group were administrated intrathecally with 0.26 μM CGRP (1161, Tocris Bioscience, Bristol, UK). Assessment of the pain threshold was done at 1 day before and 1, 4, 7, 14, 21 and 28 days after intrathecal treatment. Then the L5/6 IVDs and the L3–L5 spinal cord segments were harvested for Western blot, immunohistochemical and immunofluorescent analysis.

2.1.3. Intrathecal catheter implantation

The rats received intrathecal catheterization 5 days prior to the drug administration. Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). A polyethylene catheter (PE-10; inner diameter, 0.28 mm; outer diameter, 0.61 mm; Smiths Medical International Ltd., UK) was inserted from L5-L6 spinous processes to the lumbar enlargement, and then the outer part of the catheter was fixed onto the skin upon wound closure. An intrathecal injection of 2% lidocaine (10 μl) was performed to confirm the success of catheterization.

2.1.4. Behavioral analysis

All behavioral testing was performed by an experienced blinded investigator. Rats were habituated to the testing environment for at least 2 days before baseline testing. The mechanical pain threshold was assessed using von Frey filaments [25] (Stoelting, Wood Dale, IL, USA). The animals were placed in a Plexiglas box (26 cm × 20 cm × 14 cm) on an elevated wire mesh grid. A series of filaments with logarithmically incremental stiffness (1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g) were applied to the middle of plantar surface of the hind paw by using the up-down method [26]. Rapidly withdrawal, shrinking or flinching of the hind paw was regarded as a positive response. The minimum force required to induce positive responses in at least 3 out of 5 applications was defined as the paw withdrawal threshold (PWT).

The thermal pain threshold was assessed using the IITC Life Science Inc (Woodland Hills, CA, USA) [27].

. Rats were placed on a glass plate in the Plexiglas box, and a beam of radiant heat was applied to the plantar surface of the hind paw. The cutoff of 20 s was set to avoid thermal injury. The time from the commencement of heating to paw withdrawal was recorded. Each rat was measured three times at intervals of 10 min and the paw withdrawal latency (PWL) was calculated as the average of the three repeated measurements.

2.1.5. Histological examination

The L5/6 IVDs were decalcified in 0.05% EDTA for 4 weeks and embedded in paraffin. The samples were cut into 4-μm sections in the sagittal plane and stained with hematoxylin and eosin (H&E). The slides were observed using a digital microscope (Olympus, Japan), and the images obtained were used to assess disc degeneration.

2.1.6. Immunohistochemistry

The immunohistochemical observations were assessed according to a previously reported method [28]. Briefly, after overnight

incubation at 4 °C with the primary antibody, the sections were incubated with a biotinylated secondary antibody for 15 min at 37 °C. The primary antibody included anti-NLRP3 antibody (NBP2-12446; 1:50; Novus Biologicals, Littleton, CO, USA) and anti-CGRP antibody (ab36001; 1:200, Abcam, Cambridge, USA). Peroxidase reaction was visualized using a solution of diaminobenzidine (DAB). The mean optical density (MOD) was measured for quantitative immunohistochemical analysis using the software package Image Pro Plus (version 5.0.1, Media Cybernetics, Silver Spring, MD, USA).

2.1.7. Immunofluorescence

Spinal cords were stained with goat anti-IBA-1 antibody (ab5076; 1:200; Abcam, Cambridge, USA). Following overnight incubation at 4 °C with primary antibody, sections were incubated with donkey anti-goat Alexa 488 fluorescent antibody (ab150129; 1:1000; Abcam, Cambridge, USA) for 1 h at room temperature. Sections were examined with a fluorescence microscope.

2.2. In vitro experiments

2.2.1. Microglial cell culture and treatment

The murine microglial cell line BV2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/mL of penicillin/streptomycin at 37 °C with 5% CO_2.

LPS (Sigma–Aldrich, St Louis, MO, USA) and CGRP (Tocris Biosciences, Bristol, UK) were dissolved in sterile PBS for use. To examine whether CGRP inhibits LPS-induced microglia activation by inhibiting NLRP3 pathways, cells were pretreated for 4h with 0.5 μg/ml CGRP, followed by 0.1 μg/ml LPS for 24 h. The control group was treated with PBS alone (CON group).

2.2.2. Enzyme-linked immunosorbent assay (Elisa)

Cells were seeded into 6-well plates (2×10^6 cells/well) for protein expression, and cell-free supernatant was collected after treatment. The levels of the secreted proteins IL-1β, IL-6, and TNF-α in BV2 culture were determined using Elisa kits (MultiSciences, Hangzhou, China) according to the manufacturer's instructions.

2.2.3. Western blot analysis

Cells were lysed on ice with RIPA Lysis Buffer (Beyotime Biotech, Shanghai, China). Lysates were centrifuged at 12,000×*g* for 15 min at 4 °C, and the supernatants were collected. Proteins were separated using SDS-PAGE gel and then transferred to polyvinylidine diluoride membrane (0.22 μm, Millipore, USA). The membrane was treated with primary antibodies for IL-1β (ab9722; 1:1000;

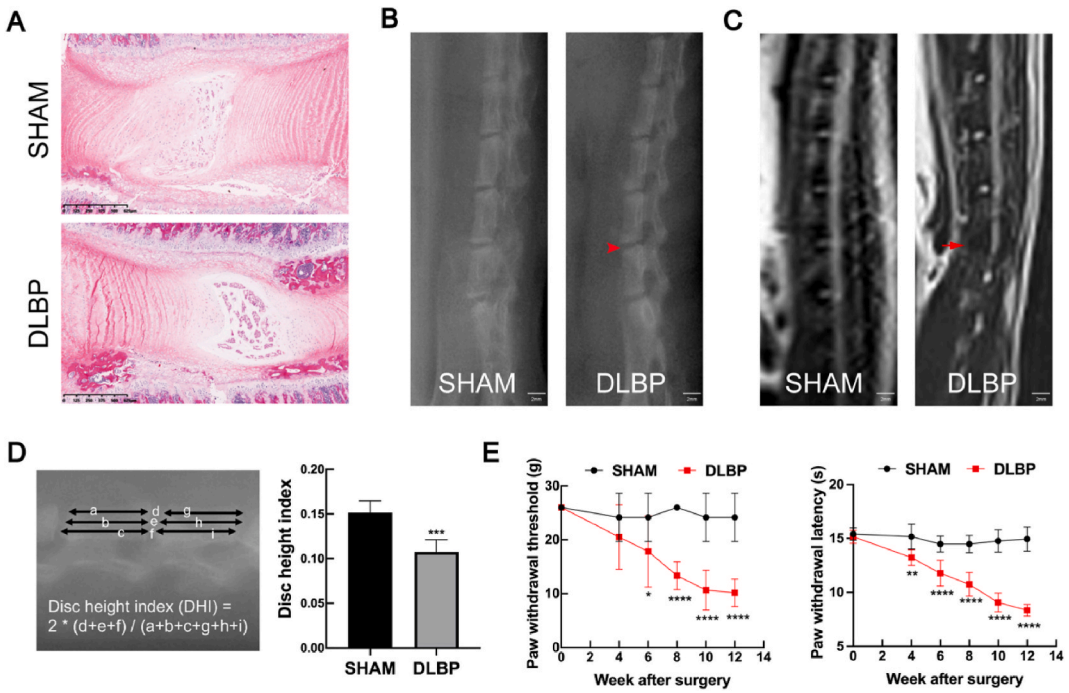

Fig. 2. Verification of the DLBP rat model. A H&E staining showed less nucleus pulposus volume, moderate serpentine pattern in annulus fibrosus and new bone formation in cartilage endplate in DLBP group (× 40). **B** X-ray images of the rat lumbar spine at 12 weeks after surgery. DLBP group showed disc height loss and disc space narrowing (arrow head). **C** T2-weighted MRI images of the spine at 12 weeks after surgery. The arrow indicates the "black disc". **D** Quantitative analysis of the disc height index between groups. **E** Behavioral analysis for mechanical and thermal pain threshold. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 versus the SHAM group.

Abcam, Cambridge, USA), IL-6 (66146-1-Ig; 1:1000; Proteintech, Chicago, IL, United States), TNF-α (60291-1-Ig; 1:1000; Proteintech, Chicago, IL, United States), caspase-1 (22915-1-AP; 1:1000; Proteintech, Chicago, IL, United States), NLRP3 (NBP2-12446; 1:1000; Novus Biologicals, Littleton, CO, USA), GAPDH (10494-1-AP; 1:1000; Proteintech, Chicago, IL, United States) or β-tubulin (10094-1-AP; 1:1000; Proteintech, Chicago, IL, United States) at 4 °C overnight and then incubated for 1 h at room temperature using Horseradish peroxidase-conjugated secondary antibody (SA00001-1, SA00001-2; 1:10000; Proteintech, Chicago, IL, United States). The immunoblots were visualized using enhanced chemiluminescence (Thermo Scientific) and images were collected using ChemiDoc XRS (Bio-Rad, USA). Bands were quantified using Image Lab software (Bio-Rad, USA).

2.3. Statistical analysis

All experiments and analysis were performed under blinded conditions. Data were analyzed using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). All data were presented as the means ± standard deviation. The statistical significance between two groups was evaluated by the Student's *t*-test. One-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for the analysis of the comparisons among multiple groups. The significance level was set at $P < 0.05$.

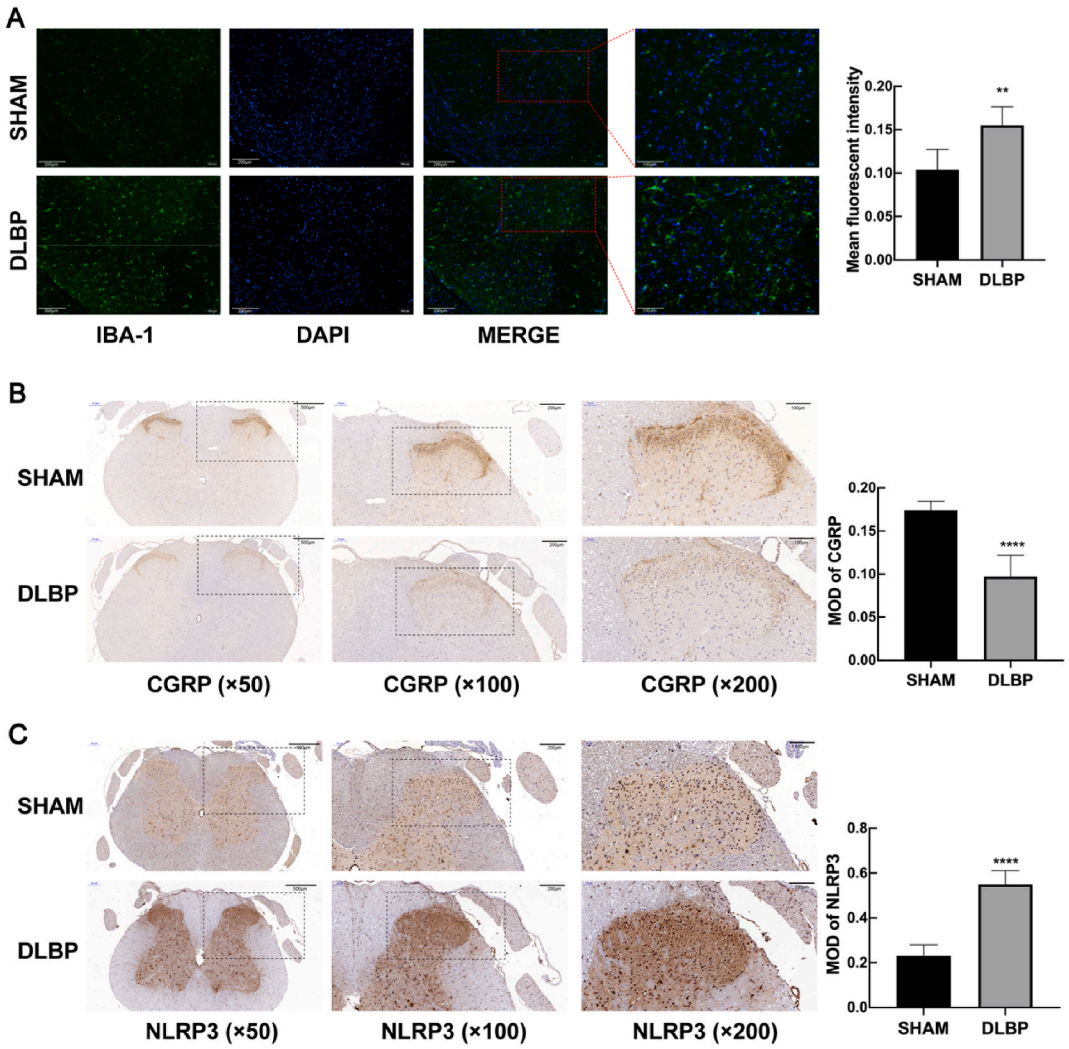

Fig. 3. Expression of IBA-1, CGRP and NLRP3 in spinal cord. A Representative images and quantitative analysis of immunofluorescence staining with IBA-1 in spinal cord from rats at 12 weeks after surgery (× 100; × 200). The data are expressed as the means ± SD (n = 6 in each group). **B** Immunohistochemical staining for CGRP in spinal cord (× 50; × 100; × 200) and quantitative analysis at 12 weeks after surgery. **C** Immunohistochemical staining for NLRP3 in spinal cord at 12 weeks after surgery (× 50; × 100; × 200) and quantitative analysis. The data are expressed as the means ± SD (n = 6 in each group). ****P < 0.0001 versus the SHAM group.

3. Results

3.1. Verification of the DLBP rat model

At 12 weeks postoperatively, the lumbar IDD was assessed through histology and radiography. As shown in Fig. 2A, the disc of DLBP group showed less nucleus pulposus volume, moderate serpentine pattern in annulus fibrosus and sclerotic in cartilage endplate, where part of cartilage was replaced with new bone formation. Lateral radiographs of the DLBP group showed degenerative changes, including disc height loss and disc space narrowing (Fig. 2B). T2-weighted MRI images displayed a substantial reduction in the signal intensity of the nucleus pulposus in the DLBP group at 12 weeks after surgery (Fig. 2C).The disc height index (DHI) [29] was calculated from lateral radiographs as the ratio of the average anterior, middle, and posterior disc height to the average of the adjacent vertebral body heights. As shown in Fig. 2D, the DHI in the DLBP group was significantly decreased when compared with that in SHAM group.

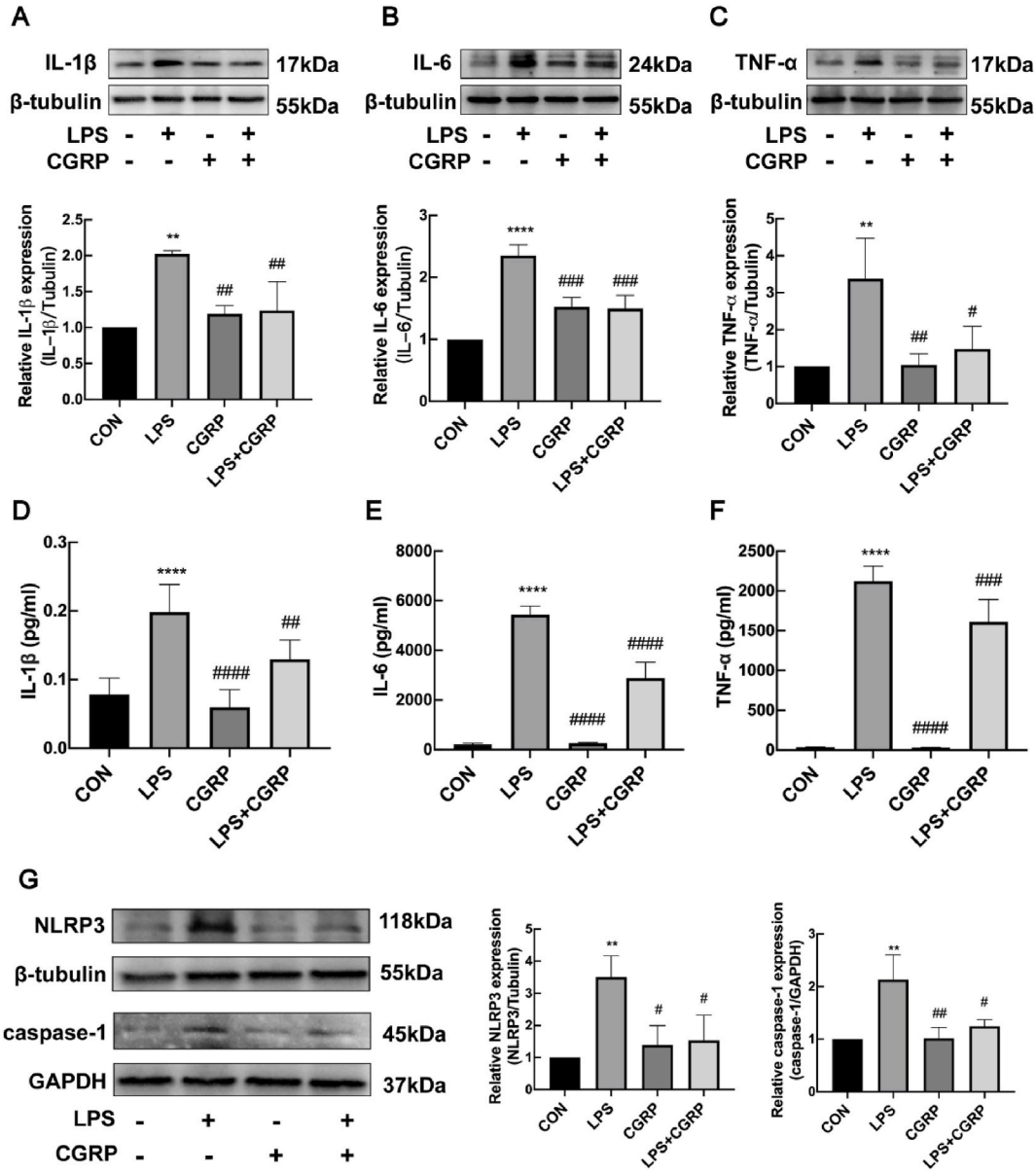

Fig. 4. CGRP treatment in vitro inhibited activation of microglial cells and NLRP3/caspase-1 induced by LPS. A-C Representative blots and quantification of IL-1β, IL-6 and TNF-α in BV2 cell. **D-F** Elisa assay for secreted protein levels of IL-1β, IL-6 and TNF-α of BV2 cells. **G** Representative blots and quantification of NLRP3 and caspase-1 in BV2 cell. The original blots were provided in supplementary data. The data are expressed as the means ± SD (n = 3 in each group). **P < 0.01; ****P < 0.0001 versus the CON group. $^{\#}$P < 0.05; $^{\#\#}$P < 0.01; $^{\#\#\#}$P < 0.001; $^{\#\#\#\#}$P < 0.0001 versus the LPS group.

The results of the behavioral assessment for mechanical and thermal sensitivity are presented in Fig. 2E. The mechanical PWT and thermal PWL were both decreased from weeks 4–12 in the DLBP group, compared with baseline. And compared to SHAM rats, the DLBP rats exhibited a decreased PWT from weeks 6–12, and a decreased PWL from weeks 4–12. These results indicated that the DLBP model was successfully induced by unbalanced dynamic and static force surgery.

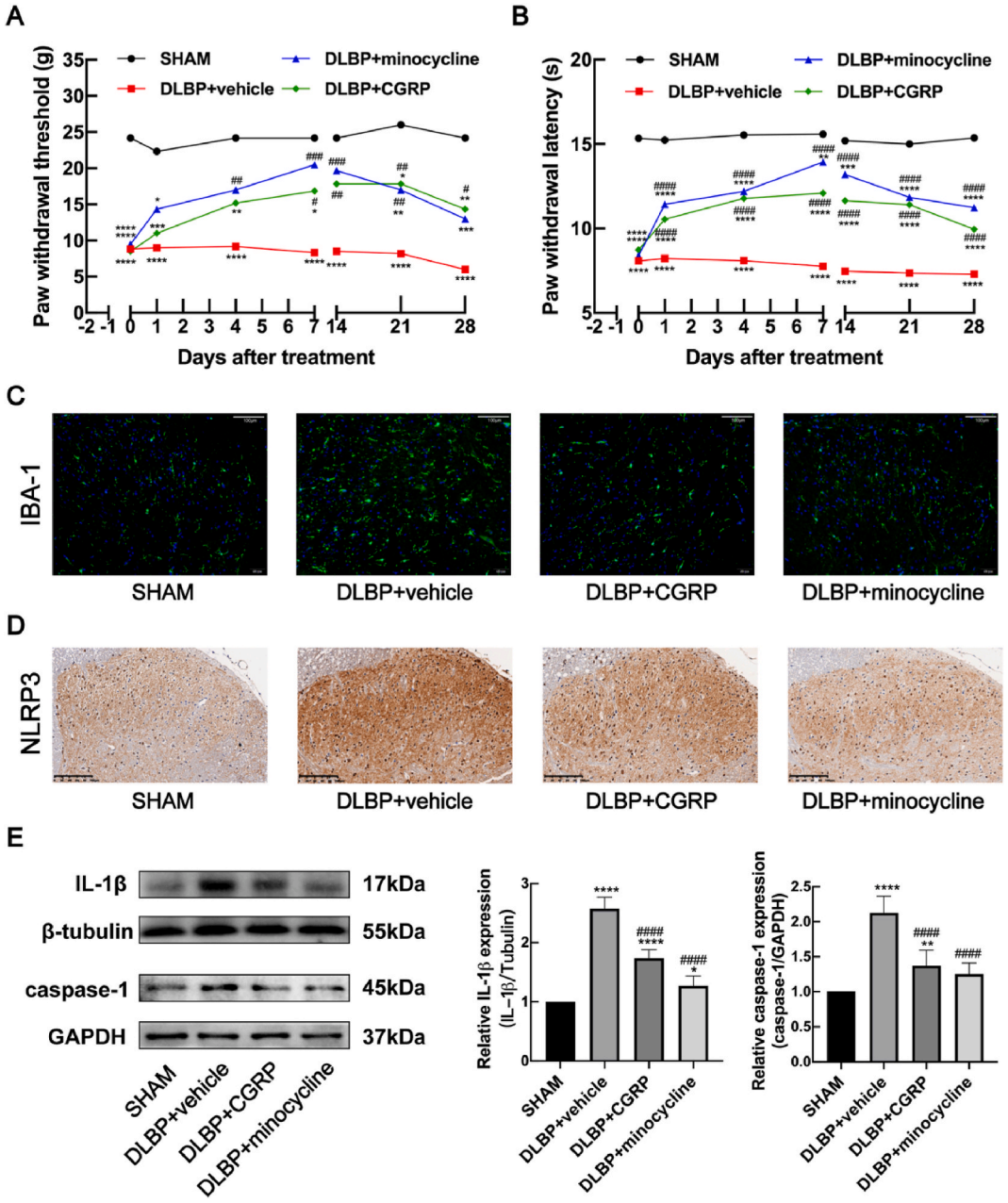

Fig. 5. CGRP treatment in vivo relieved hyperalgesia and inhibited activation of microglia and NLRP3/caspase-1 in DLBP rats, similar to minocycline A-B Behavioral analysis for mechanical and thermal pain threshold was performed at days 0, 1, 4, 7, 14, 21 and 28 after intrathecal injection. **C** Representative images of immunofluorescence staining with IBA-1 in spinal cord from rats at days 28 after intrathecal injection (× 200). **D** Representative images of immunohistochemical staining with NLRP3 in spinal cord from rats at days 28 after intrathecal injection (× 200). **E** Representative blots and quantification of caspase-1 and IL-1β in spinal cord from rats. The original blots were provided in supplementary data. The data are expressed as the means ± SD (n = 6 in each group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 versus the SHAM group. $^{\#}$P < 0.05; $^{\#\#}$P < 0.01; $^{\#\#\#}$P < 0.001; $^{\#\#\#\#}$P < 0.0001 versus the DLBP + vehicle group.

3.2. Microglia activation and changed expression of CGRP and NLRP3 in spinal cord of DLBP rats

Microglial activation in the spinal dorsal horn was investigated by immunofluorescent labeling of IBA-1. The expressions of CGRP and NLRP3 in the spinal cord were examined by immunohistochemical analysis. As shown in Fig. 3A, the number of IBA-1-positive cells in the spinal cord were significantly increased in DLBP rats than that in SHAM rats. The immunohistochemical analysis showed that DLBP rats exhibited reduced expression of CGRP and increased expression of NLRP3 in the spinal cord (Fig. 3B and C).

3.3. CGRP treatment in vitro inhibited activation of microglial cells and NLRP3/caspase-1 induced by LPS

LPS is a widely used proinflammatory agent and is known to activate microglia. To investigate whether CGRP can suppress microglial activation in LPS-induced BV2 cells, we examined the expression levels of proinflammatory cytokines using western blotting (Fig. 4A–C) and Elisa assay (Fig. 4D–F), including IL-1β, IL-6, and TNF-α. The results showed that LPS triggered microglial activation and upregulated the expression of IL-1β, IL-6, and TNF-α. Following CGRP treatment, there was a prominent reduction in the levels of these proinflammatory cytokines. As shown in Fig. 4G, CGRP treatment significantly suppressed the upregulated protein expression of NLRP3 and caspase-1 induced by LPS. Intriguingly, CGRP alone showed little effect on the protein levels of proinflammatory cytokines and NLRP3/caspase-1. These findings suggested that CGRP can inhibit LPS-induced microglial and NLRP3/caspase-1 pathway activation.

3.4. CGRP treatment in vivo relieved hyperalgesia and inhibited activation of microglia and NLRP3/caspase-1 in DLBP rats, similar to minocycline

This part of experiment was designed to focus on the effects and mechanism of CGRP on the hyperalgesia in vivo. To investigate the role of microglia activation in this process, we employed the minocycline, an inhibitor of microglial activation, as a control. CGRP or minocycline were intrathecally injected once daily for 7 days at 12 weeks after surgery. As shown in Fig. 5A and B, PWT in DLBP rats was significantly increased after intrathecal injection of minocycline from day 4, and increased after intrathecal injection of CGRP from day 7. PWL in DLBP rats was significantly increased after intrathecal injection of minocycline or CGRP from day 1. After stopping the CGRP or minocycline administration from day 7, the PWT and PWL gradually decreased over time. Importantly, immunofluorescence showed that compared with the DLBP + vehicle group, the expression of IBA-1 in spinal cord was significantly decreased in the DLBP + CGRP and DLBP + minocycline group (Fig. 5C). The NLRP3/caspase-1 pathway was examined as well. As shown in Fig. 5D, the expression of NLRP3 in spinal cord was significantly decreased in the DLBP + CGRP and DLBP + minocycline group, when compared with DLBP + vehicle group. And Western blot analysis showed that the expression of caspase-1 and IL-1β were significantly down-regulated in the spinal cord after CGRP or minocycline treatment (Fig. 5E). These results demonstrated that CGRP and minocycline could inhibit microglial activation and NLRP3/caspase-1 pathway in the spinal cord, and relieved hyperalgesia in DLBP rats.

4. Discussion

To date, the mechanisms underlying chronic DLBP are not fully understood. In this study, we investigated the role of CGRP in DLBP induced by unbalanced dynamic and static forces. The current study demonstrated three novel findings using a DLBP rat model. First, rats with unbalanced dynamic and static force presented significant hyperalgesia and IDD, which are well-suited as a model for DLBP study. Second, microglia were found to be activated in the spinal cord of DLBP rats, with decreased expression of CGRP and increased expression of NLRP3. Third, CGRP relieved mechanical and thermal hyperalgesia in DLBP rats, inhibited the microglial activation and decreased the expression of NLRP3/caspase-1 in spinal cord. Our results suggested that CGRP may play an antinociceptive role in DLBP via the inhibition of microglial activation in the spinal cord.

To investigate the mechanisms of DLBP, we first employed the unbalanced dynamic and static force rat model, which was usually used in the IDD researches [23]. Compared with other methods, such as disc puncturing, the surgery we used induces IDD via biomechanical disturbance instead of directly injuring the intervertebral disc. The radiological and histological examinations both identified the degeneration of the lumbar intervertebral disc in rats at 12 weeks postoperation. On the other hand, the rats received surgery exhibited significant mechanical and thermal hyperalgesia. These findings confirmed that the DLBP was successfully induced by unbalanced dynamic and static force surgery.

Microglia are the resident immune cells of the CNS, which can be activated by stimuli that threaten physiological homeostasis [8]. Activated microglia release various nociceptive mediators including pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6. The pro-inflammatory cytokines interact with nociceptive neurons and modulate central sensitization and hyperalgesia [30,31]. Within the activated NLRP3 inflammasome complex, the pro-IL-1β is cleaved by active caspase-1 to be secreted in the active form thereby amplifying the inflammatory response [32,33]. He et al. [15] demonstrated that blockade of microglial NLRP3/IL-1β could improve hyperalgesia in migraine model, and inhibit the increase in biomarkers related to central sensitization. The activation of microglial NLRP3/IL-1β has been found to account for the long-term morphine treatment induced analgesic tolerance and hyperalgesia [34]. The $NLRP3^{-/-}$ mice showed a higher nociceptive threshold and decreased analgesic tolerance induced by morphine [35]. These finding indicated that the microglial NLRP3/IL-1β plays critical roles in the induction of hyperalgesia. In the present study, the microglia in spinal cord were found significantly activated in DLBP rats, with the increase of NLRP3, caspase-1 and IL-1β protein expression. Our results suggested a potential role of microglial NLRP3/caspase-1/IL-1β pathway in hyperalgesia in the DLBP.

CGRP is an important neuropeptide that exerts complicated effects in pain modulation and transmission in peripheral nervous

system and CNS [10]. CGRP expressed in DRG has always been reported to exert pronociceptive effects. Sato et al. [36] reported that CGRP-IR in DRG neurons increased following disc injury, and the upregulation of CGRP might induce pain. The release of CGRP from the trigeminal ganglion is considered as a trigger of migraine. In addition to conventional therapeutic agents such as triptans, CGRP-targeted therapy including CGRP receptor antagonists and anti-CGRP antibodies have proved effective for migraine pain relief [37,38]. These treatments usually do not cross the blood brain barrier. Apart from the pronociceptive effects, CGRP also induced an antinociceptive effect in many brain areas, including the periaqueductal grey (PAG), nucleus accumbens (NAc), and the central nucleus of amygdala (CeA) [10], which are crucial structures and nuclei involved in pain transmission and modulation. For example, PAG acts as way station for the signal of analgesia in the descending pathway from brain to dorsal horn of the spinal cord [39,40], and CGRP induces significant analgesia through CGRP receptor 1 in PAG [41]. Furthermore, it was reported that CGRP-ergic nerve fibers from nucleus raphe magnus (NRM) run along the dorsal longitudinal tract and enter the spinal cord, resulting in postsynaptic inhibition on the pain transmission [40]. Also, CGRP induces dose-dependent antinociception through CGRP receptor 1 in NRM [42]. Therefore, the mechanisms underlying regulations of pain by CGRP remain controversial. Studies have shown that CGRP mediated protective effects in inflammation such as allergic airway inflammation [43] and lung injury [44]. Duan et al. [20] reported that CGRP inhibited the LPS-induced activation of macrophages by reducing the expression of NLRP3 and IL-1β. Xu et al. [21] demonstrated that CGRP alleviated the inflammatory reaction in mice keratitis and reduced the levels of NLRP3 and IL-1β in A. fumigatus stimulated-macrophages. And results from a previous study suggested that CGRP exerted a potent inhibitory effect on LPS-induced microglia activation in vitro, while the underlying mechanisms remain unknown [22]. In the present study, we observed the decreased expression of CGRP in the spinal cord of DLBP rats, while the underlying mechanisms are still unclear. CGRP in the spinal cord is considered to be released from afferents [45]. Studies showed that the level of CGRP in spinal cord would be down-regulated in several types of nerve injury such as crush and ligation [11,46], which may be associated with the blockade of axonal transport. Therefore, we speculate that the reduction of CGRP in spinal cord of DLBP rats might be attributed to the mechanical compression of afferents during spine degeneration induced by unbalanced dynamic and static forces. To further explore the roles of CGRP in the microglial activation and the underlying mechanism, we performed in vitro experiments with BV2 microglia cells. We found that CGRP treatment significantly inhibited LPS-induced microglia activation in vitro, which is consistent with the previous study [22]. Further, the increase in the protein expression of NLRP3 and caspase-1 induced by LPS was inhibited by CGRP as well. The findings suggest an inhibitory effect of CGRP on LPS-mediated microglia activation and NLRP3/caspase-1/IL-1β pathway in vitro. CGRP has also been reported to suppress LPS-induced expression of NLRP3/caspase-1 and inflammatory cytokines in some other cells such as macrophages and intestinal epithelial cells [20,21,47], and Ning et al. have demonstrated that CGRP acts through inhibiting the NF-κB pathway [47]. However, the upstream mechanisms for example the target receptor of CGRP in BV2 cells, remain unknown. BV2 cells have been shown to express CGRP receptor components [48]. Further research is necessary to investigate whether CGRP inhibits microglia activation through direct effects (CGRP or other receptors) or indirect effects.

For the validation of the in vitro results, the in vivo study in DLBP rats was designed in the present study. Minocycline is a common microglial inhibitor with strong analgesic and anti-inflammatory properties under a variety of pain models, such as neuropathic pain [49], inflammatory pain [50], and bone cancer pain [51]. Lu et al. [52] reported that the pretreatment of minocycline significantly inhibited NLRP3 inflammasome activation in BV2 microglial cells. In the current study, the minocycline and CGRP were intrathecally injected into DLBP rats, respectively. Consistent with the other pain models, in the DLBP model, the hyperalgesia was significantly alleviated following the administration of minocycline. Additionally, as expected, the minocycline treatment inhibited microglial activation in the spinal cord, and downregulated the expression levels of NLRP3, caspase-1 and IL-1β. Similarly, the intrathecal injection of CGRP alone significantly attenuated nociceptive behavior in the DLBP rats. The inhibitory effect on microglial activation and NLRP3/caspase-1/IL-1β signaling in the spinal cord has also been observed following the administration of CGRP. However, whether CGRP directly or indirectly inhibit microglia activation in spinal cord remains unknown. And future studies could also examine if combination therapies with CGRP and minocycline could provide additional efficacy on DLBP. Our results are inconsistent with those from other studies reporting pronociceptive roles for CGRP in the spinal cord [53,54]. DLBP is a complex and multi-factorial disease, and the underlying mechanisms are still unclear. One difference in the DLBP model is that an decreased expression of CGRP in spinal cord is observed in DLBP models, which is usually increased in some other pain models [55,56]. The reduced CGRP in spinal cord was also observed in some peripheral nerve injury models, in which CGRP was not able to be transported to the spinal cord [11,57]. One possible future research direction is to investigate whether the transportation of CGRP to spinal cord is affected in DLBP model. On the other hand, with the intrathecal administration, the drugs could be delivered to the CNS including brain parenchyma. In this case, CGRP may also produce the antinociception by acting at the regions in addition to spinal cord. In vivo pain modulation is rather complex and involves multiple pain processing regions and co-regulators such as glia, neurons and neuropeptides. Our results suggest that the ability of minocycline and CGRP to alleviate hyperalgesia in DLBP rats may be ascribed, at least in part, to an inhibition in microglial activation and NLRP3/caspase-1/IL-1β signaling in the spinal cord. Yet, further studies are warranted to determine whether other factors like astroglia are involved in this process.

Several limitations to this study need to be acknowledged. First, we examined a wide range of time points for the behavioral tests, while the immunohistochemical and Immunofluorescence analysis was only performed at the last time point during in vivo experiments. Thus the changes of the microglia in the spinal cord could not be observed dynamically and matched with the pain thresholds in this study. Second, in the in vivo experiments we did not set different doses and courses of CGRP, therefore it is still not clear whether or not the analgesic effect of CGRP depends on the concentration and duration. Third, the results in this study could not support a direct action of CGRP on microglia in vivo, and the mechanisms underlying CGRP inhibiting microglia activation remain unclear. In spite of its limitations, the study certainly adds to our understanding of the DLBP pathogenesis as well as the treatment of this disease.

5. Conclusions

In conclusion, our results suggest that microglial activation in the spinal cord play a key role in hyperalgesia in DLBP rats. Both CGRP and minocycline alleviate hyperalgesia and inhibit microglial activation in the spinal cord in DLBP rats. Our study provides several new insights for the development of novel therapeutic strategies for DLBP.

Ethics statement

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, with the approval number: [RJ2018-1012].

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Weixin Xie: Writing – original draft, Methodology, Investigation. **Fan Li:** Writing – original draft, Methodology, Investigation. **Yi Han:** Data curation. **Xiaoying Chi:** Data curation. **Yi Qin:** Software, Resources. **Fan Ye:** Software, Resources. **Zhanchun Li:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Jie Xiao:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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