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Lycium Barbarum Glycopeptide alleviates neuroinflammation in spinal cord injury via modulating docosahexaenoic acid to inhibiting MAPKs/NF-kB and pyroptosis pathways.

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ABSTRACT

Background: Lycium barbarum polysaccharides (LBP) is an activity ingredient extracted from Lycium barbarum, which has the effect of inhibiting neuroinflammation. Lycium Barbarum Glycopeptide (LbGp) is a glycoprotein with immunological activity that is further purified and isolated from LBP. Previous studies have shown that LbGp can regulate the immune microenvironment, but its specific mechanism of action is still unclear.

Aims: In this study, we aim to explore the mechanism of action of LbGp in the treatment of spinal cord injury through metabolomics and molecular experiments.

Methods: SD male rats were randomly assigned to three experimental groups, and after establishing the spinal cord hemisection model, LbGp was administered orally, and the spinal cord tissue was sampled on the seventh day after surgery for molecular and metabolomic experiments. In vitro, LbGp was administered to mimic the inflammatory microenvironment by activating microglia, and its mechanism of action in suppressing neuroinflammation was further elaborated by metabolomics and molecular biology techniques such as western blot, q-PCR.

Results: Through in vivo and in vitro experiments, it was found that LbGp can improve the inflammatory microenvironment by inhibiting the NF-kB and pyroptosis pathways. Furthermore, it was found that LbGp can induce the secretion of docosahexaenoic acid (DHA) by microglia, and DHA can inhibit neuroinflammation through the MAPKs/NF-kB and pyroptosis pathways.

Conclusions: In summary, we believe that LbGp improves the inflammatory microenvironment by regulating the secretion of DHA in microglia, thereby inhibiting the MAPKs/NF-kB and pyroptosis pathways.
promoting nerve repair and motor function recovery. This study provides a new direction for the treatment of spinal cord injury and elucidates the potential mechanism of action of LbGp.

Introduction

Spinal cord injury (SCI) is a highly disabling and lethal disease of the central nervous system (CNS) that lacks effective treatment strategies. The incidence of SCI in China has increased in recent years owing to various factors, such as tumors, hemorrhage, inflammation, and trauma [1], there approximately 17000 new patients with SCIs reported annually across the United States [2]. Microglia are immune cells in the CNS that play a critical role in neuroinflammation, homeostasis, and stress in vivo [3]. Cytokine production mediated by overactivated microglia after SCI triggers an extensive inflammatory cascade, with substantial production of inflammatory factors and reactive oxygen species (ROS) exacerbating the secondary injury, chemokines recruiting peripheral immune cells into the injured area, and reactive nitric oxide species, damage-related molecular patterns (DAMP), and inflammatory signals that induce their entry into the tissues surrounding the injured area, thereby contributing to the clearance of pathogens and cellular debris [4-6].

Activation of the cytoplasmic inflammasome is a fundamental step in neuroinflammatory processes and a key trigger for neuronal pyroptosis [7]. To date, five receptor proteins have been confirmed to assemble inflammasomes, including the nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing protein (NLR)family members NLRP1, NLRP3, and NLRP4, as well as proteins absent in melanoma2 (AIM2) and pyrin [8]. SCI reportedly triggers NLRP3 inflammasome activation in spinal cord microglia [9]. The NOD-like receptor protein-3 (NLRP3) inflammasome, assembled from NLRP3, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), endogenous “danger signal” followed by cysteine protease-1, and exogenous infection, is an important cytosolic protein complex [10]. The activation of the NLRP3 inflammasome requires two key steps and an activation step, in which the binding of DAMP and pathogen-associated molecular patterns to pattern recognition receptors activates the toll-like-receptor (TLR)-myeloid differentiation primary response protein MyD88-nuclear factor-kB (NF-kB) pathway to induce inflammasome transcription and trigger post-translational modification. The second step is the assembly and activation of the inflammasome, and inflammasome activation can be achieved through three mechanisms: ROS activation by NADPH oxidase, lipopolysaccharide (LPS) and ATP production, lysosomal rupture, and ion channel gating; NADPH oxidase-mediated ROS play a vital role in this process[7, 11-13]. Activated NLRP3 nucleates the pyroptosis-associated apoptotic protein-containing caspase activation and recruitment domain (CARD) helical fibrils to form ASC patches, which recruit and activate pro-caspase-1 cleavage and activation of pro-interleukin (IL)-1β, pro-IL-18, and GSDMD [11]. Cleavage of the N-terminal end of GSDMD drives pyroptosis, which leads to cell death and allows cells to release mature IL-1β and IL-18 [8, 14].

LbGp is a glycoprotein with immunological activity that is further purified and isolated from LBP [15]. Following metabolomic analysis (Ningxia Tianren GoJi Biotechnology, Ningxia, China), we have previously revealed that LbGp comprises 368 metabolites, including 42 others (40%), 118 terpenoids (32%), 38 alkaloids (10%), 24 phenylpropanoids (6.5%), 17 phenols (4.6%), 17 flavonoids (4.6%), 4 amino acids (1.1%), 3 aliphatic acyl (0.8%), and 3 fatty acids (0.8%). However, the precise LbGp component that improves the inflammatory microenvironment in vivo by inhibiting MAPKs- NF-kB and pyroptosis-related pathways needs to be identified.

In the present study, we aimed to establish that LbGp induces microglia to secrete DHA in vivo to...
promote nerve regeneration and motor function repair and improve the inflammatory microenvironment by inhibiting MAPKs-NF-kB, and pyroptosis-related pathways, providing a new direction for the treatment of SCI. Accordingly, we performed in vivo and in vitro molecular and metabolite analyses experiments.

2. Methods and materials

2.1 Animals

In total, 70 (8-weeks old) male Sprague-Dawley rats (300-350 g) were purchased from the Laboratory Animal Center Ningxia Medical University. After surgery, all rats were housed under continuous temperature (21±3°C) and humidity (50%±5%) with a 12-h light/dark cycle. The experimental protocol was approved by the Laboratory Animal Ethical and Welfare Committee of the Laboratory Animal Center of Ningxia Medical University.

2.2 Rats spinal cord injury model and treatments groups

The twelfth thoracic vertebral body (T12) hemisection model was used as previously described [16]. To establish the SCI model, rats were anesthetized with isoflurane (2-4% induction, 1.5% maintenance), and body temperature was maintained at 35±1°C using a mini heating pad. The skin of the upper thoracic area was exposed after shaving and cleaning with betadine solution. To expose T12, the fascia and muscle were bluntly dissected to avoid spinal dural injury during the incision of the dorsal lamina. A needle (26G) was bent by 90° to establish a lateral hemisection, and the spinal cord was punctured dorsoventrally at the middle line by inserting the needle 5 mm while averting damage to the dorsal spinal artery. The left half of the spinal cord was pulled and cut, repeated three times to ensure completeness of the spinal cord hemisection (Fig.S2A-B). For pain management, carprofen (4 mg/kg; Rimadyl®, Zoetis, Florham Park, NJ) and buprenorphine (0.2 mg/mL; Temgesic®, Intervet International, Boxmeer, the Netherlands) were administered continuously before surgery and every 8 h for three successive days after SCI.

Forty-five adult male rats were randomly assigned to Three groups (15 rats per group): sham group; SCI group; post-SCI LbGp treatment group; LbGp dissolved in 0.01M phosphate buffer saline at room temperature; LbGp administered via a nasogastric tube (50 mg/kg) once daily until day 28 post-SCI. The sham, SCI groups were administered 0.01M phosphate buffer saline 24 h after SCI.

2.3 Behavioral testing

We next evaluated hind limb motor function in rats after SCI. Three rats were selected from each group, and the Basso-Beattie-Bresnahan (BBB) test was used to score the motor function of the hind limbs. The BBB test is divided into 22 grades, with 21 points indicating normal paralysis and 0 points indicating paralysis. The scoring was performed at 8 p.m. using a double-blind method. Each rat was measured three times, and the average value was recorded.

2.4 Western blot

The rat microglia (RM) cell line (BNCC 360237) was purchased from the BeNa culture collection (Henan, China). First, RM cells were seeded in six-well plates (1×10^5 cells/well) and activated with LPS (1 µg/ml L3129 Sigma) and ATP (5 mM; Sigma) for 4 h, followed by treatment with LbGp or DHA for 24 h to establish the LbGp treatment group or DHA treatment group. In addition, microglia activated with ATP+LPS were incubated with the FADs2 enzyme inhibitor SC26196 for 12 hours and then incubated with LbGp for 24 hours to construct the ATP+LPS+ SC26196+ LbGp treatment group. Spinal cord tissue
was harvested on day 7 post-SCI. Tissue or cell lysates were prepared using the Keygen protein extraction kit (KGP250 Keygen BioTECH China), and the protein concentration was measured with the BCA protein assay kit (KGP902 Keygen BioTECH China). Equal amounts of protein were separated with 10 or 15% sodium dodecyl sulfate-polyacrylamide gel, transferred to polyvinylidene fluoride membranes, and blocked with 5% skim milk. Subsequently, the membranes were probed with the following primary antibodies at 4°C overnight: anti-brain-derived neurotrophic factor (BDNF) (1:10000) (Cat. Number: ab108319; Abcam UK), anti-glia-derived neurotrophic factor (GDNF) (1:2000) (Cat. Number: A14639; ABclonal China), anti-P-p-38 (1:1000) (Cat. Number:28796-1-AP; Proteintech, China), anti- p38 (1:1000) (Cat. Number: 14064-1-AP; Proteintech, China), anti-P-p-JNK (1:1000) (Cat. Number: 80024-1-RR; Proteintech, China), anti- JNK (1:1000) (Cat. Number:24164-1-AP; Proteintech, China), anti-p-P65 (1:1000) (Cat. Number: ab76302; Abcam, Cambridge, UK), anti-p65 (1:20000) (Cat. Number: 80979-1-RR; Proteintech, China), anti-NLRP3 (1:2000) (Cat. Number: 27458-1-AP; Proteintech, China), anti-ASC (1:3000) (Cat. Number:10500-1-AP; Proteintech, China), anti-Caspase-1 p45 (1:1000) (Cat. Number: ab179515; Abcam UK), anti-Caspase-1 p20 (1:2000) (Cat. Number: bs-10442R; Bioss, China), anti-GSDMD and anti-GSDMD-N (1:1000) (Cat. Number: ab219800; Abcam, UK), anti-Pro-IL-18 and anti-IL-18 (1:10000) (Cat. Number:10663-1-AP; Proteintech, China), anti-IL-1β (1:1000) (Cat. Number: ab254360; Abcam, UK), anti-β-actin (1:40000) (Cat. Number: 81115-1-RR; Proteintech China). The membrane was washed with TBST and incubated with a secondary antibody (1:20000) (Cat. Number: ab6721 Abcam UK) for 1 h at room temperature, washed again, visualized with ECL reagent (SW134-01 Seven Biotech, China), and quantitatively analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5 Cell viability assay

The Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories Lot.CK04 Japan) was used to assess the toxicity of LbGp toward microglia. Microglia were plated in 96-well plates at 7×10^3 cells/well, and different concentrations of LbGp were added (100, 200, and 400 µg/ml), followed by incubation at 37°C in a 5% CO₂ incubator for 24 h. Then, the supernatant was discarded, and 100 µl of the medium containing 10% CCK-8 reagent was added to each well, followed by incubation at 37°C in a 5% CO₂ incubator for 2 h in the dark. The absorbance of each well was detected under the 450 nm wavelength spectrum of a microplate reader.

2.6 Enzyme-linked immunosorbent assay analysis (ELISA)

Level of DHA in each group were detected by COIBO BIO (Shanghai China). RM cells were seeded in six-well plates (1×10^5 cells/well) and activated with LPS (1 µg/ml L3129 Sigma) and ATP (5 mM; Sigma) for 4 h, followed by treatment with LbGp for 24 h, the cell were centrifuged at 4°C, 800g for 10 min, and 200 ul of supernatant was then collected for DHA detection. DHA assays were performed according to the protocol provided by the COIBO BIO (CB14901-Ra Shanghai China).

2.7 RNA preparation and reverse transcription-quantitative PCR

Microglia were counted and plated into a six-well plate at a density of 1×10^5 cells/well. On reaching 80% confluency, cells were incubated with 1 µg/ml LPS for 12 h and then treated with different concentrations of LbGp (100, 200, 400µg/ml), followed by incubation for 24 h. After washing three times with cold phosphate-buffered saline (PBS), 1 ml of Trizol was added and pipetted into a 1.5 ml EP tube, followed by 200 µl of chloroform. The prepared samples were centrifuged at 9000×g at 4°C for 15 min. The supernatant was aspirated, and an equal volume of isopropanol was added, followed by centrifugation at...
9000×g at 4°C for 15 min. The supernatant was removed, and 75% ethanol was added, followed by centrifugation at 6000×g twice for 5 min at 4°C. The RNA concentration was measured after adding double-distilled water (ddH2O). The reverse transcription kit and real-time quantitative PCR kit were all purchased from Vazyme (lot. R302-01, Q411-02; Nan Jing, China) and performed according to the manufacturer’s instructions. Gene specific primer sequences used are shown in supplementary Table 1. The relative gene expression was measured using the 2^−ΔΔct method.

2.8 Immunofluorescence staining

Rat spinal cord tissue was collected on day 7 post-SCI, which was then subjected to fixation, paraffinization, dewaxing, dehydration, antigen extraction, and blocking, according to the standard protocol. Subsequently, tissue sections were incubated with primary antibodies against anti-NLRP3, anti-ASC, anti-Caspase-1, and anti-NeuN (1:2000) (Cat. Number:6975-1-AP; Proteintech, China) for 24 h. Next, tissue sections were incubated with fluorescent-labeled secondary antibody green (1:500) (ab150077; Abcam UK), red (1:200 SA00013-4; Proteintech, Wuhan, China), rose red (1:200 GB21303 Servicebio China), pink (1:200 GB1232 Servicebio China) for 3 h, and the nuclei were stained with DAPI. Images were scanned using an ortho-fluorescent microscope (Nikon eclipse C1) and a fluorescence microscope (Olympus, Japan). Quantitative analysis was performed using ImageJ.

2.9 Metabolite processing and identification

One-week post-SCI, three groups (A: sham group, B: SCI group, C: LbGp treatment groups; n= six rats/group) were anesthetized under excessive isoflurane, and the dorsal skin surface was incised. The vertebrae were cut, and the injured area of the spinal cord tissue was harvested (approximately 50 mm in length and approximately 30 mg in weight). The harvested spinal tissue was quickly placed in liquid nitrogen and transferred to a -80°C refrigerator for metabolomic sequencing analysis.

Metabolomics was performed by Shanghai Luming Biotech Co.Ltd. All reagents were of high-pressure liquid chromatography (HPLC) grade. L-2-chlorophenylalanine was purchased from Shanghai Heng Chuang Bio-technology (Shanghai, China), methoxyamine hydrochloride (97%), pyridine, n-hexane, and BSTFA with 1% TMCS were purchased from CNW Technologies GmbH (Düsseldorf, Germany). Chloroform was obtained from Titan Chemical Reagent (Shanghai, China), and water and methanol were obtained from Thermo Fisher Scientific (Waltham, USA).

Briefly, 30 mg of sample was added to a 1.5 ml centrifuge tube, followed by the addition of two small steel beads and 600 µl methanol-water (V:V+4:1, containing L-2-chlorophenylalanine, 4 µg/ml) and incubation for 2 min. The samples were placed in a grinder (60 Hz, 2 min); 120 µl of chloroform was added and vortexed for 2 min. Ultrasonic extraction was performed in an ice-water bath for 10 min at -40°C or for 30 min. Subsequently, centrifugation was performed for 10 min (12000×g 4°C). Next, 150 µl of the supernatant was placed in a glass derivatization bottle, and the sample was dried using a centrifugal concentrator desiccator. Next, 80 µl of methoxyamine hydrochloride pyridine solution (15 mg/ml) was added to the glass derivatized vial, and the sample was vortexed for 2 min and placed in an incubator maintained at 37°C for 60 min to perform the oxidation reaction. After removing the sample, 50 µl of BSTFA derivatization reagent and 20 µl of n-hexane were added, and 10 internal standards (c8/c9/c10/c12/c14/c16/c18/c20/c22/c24, all prepared in chloroform; 10 µl) were added, vortexed for 2 min, and reacted at 70°C for 60 min. The obtained samples were placed at room temperature for 30 min to undergo gas chromatography-mass spectrometry (GC-MS) metabolomic analysis. Quality control samples were prepared by mixing equal volumes of extracts from all samples.
GC-MS was performed using a Db-5MS capillary column (30×0.25 mm×0.25 µm; Agilent J&W Folsom, CA, USA), high-purity helium (purity ≥ 99.999%) as the carrier gas, a flow rate of 1.0 ml/min, and maintaining an injection port temperature of 260°C. The injection volume was 1 µl, and the injection was split with a solvent delay of 5 min. The program temperatures were as follows: the initial temperature of the column oven was 60°C, maintained for 0.5 min; the program temperature was increased to 125°C at 8°C/min; heated up to 210°C at 8°C/min; 270°C at 15°C/min; 305°C at 20°C/min and maintained for 5 min.

For the electron bombardment ion source: ion source temperature, 230°C; quadrupole temperature, 150°C; electron energy, 70 eV. The scanning mode was full scan mode (SCAN), with a mass scanning range of 50-500 m/z.

The collected GS/MS raw data were converted into abf format by analyzing using Analysis Base File Convert to retrieve data quickly. The data were then imported into the MS-DIAL software for characterization, MS2Dec deconvolution, peak alignment, peak identification, peak detection, wave filtering, and missing value interpolation. Metabolites were characterized based on the LUG database. The data matrix included the signal intensity, retention index, retention time, mass-to-charge ratio, sample information, and peak name for each species. After screening, all peak signal intensities were segmented and normalized for each sample according to an internal criterion of RSD >0.3. After data normalization, redundancy removal and peak merging were performed to obtain a data matrix.

The matrix was imported into R for principal component analysis to observe the overall distribution between samples and the stability of the entire analysis process. Different metabolites between groups were distinguished using Orthogonal Partial Least Squares Discriminant analysis (PLS-DA). A 7-fold cross-validation and 200-response permutation test were employed to assess the model quality. The projected importance values of the variables from the OPLS-DA model were used to rank the overall contribution of each variable to the group discrimination. Two-tailed Student’s t-test was performed to verify whether the difference in metabolites between groups was significant. Differential metabolites with variable importance of projection value >1.0 and P-value <0.05 were selected.

2.10 Statistical analysis

Data are presented as mean ± S.D. One-way analysis of variance (ANOVA) was used to assess differences in comparisons of multiple group designs. All statistical analyses were performed using GraphPad Prism (version 9.3). P<0.05 was considered statistically significant.

3. Result

3.1 LbGp promotes motor function recovery after SCI.

LbGp is purified from LBP and contains 368 metabolites (Fig.S1A). LBP is known to repair nerve regeneration and promote motor function recovery [17]. To test our conjecture, by constructing a rat spinal cord hemisection model, oral LbGp was given post-operation, and the motor function of the different treatment groups was assessed up to 28 days after surgery by using the BBB motor function score. Based on the BBB test results, the SCI group score was significantly lower than that of the sham group; the LbGp group score did not significantly differ from that of the SCI group during the first-week post-SCI but was higher than that of the SCI group during the second week (Fig.1A). These results revealed that LbGp treatment could improve motor function in rats. On day 14, the BBB motor function score in the LbGp intervention group was higher than that in the SCI group.

3.2 LbGp regulates neurotrophic factors to promote nerve...
regeneration

Previous studies have shown that BDNF and GDNF play an important role in promoting nerve regeneration[18]. To further elucidate the potential role of LbGp on nerve regeneration, we detected the expression levels of BDNF and GDNF in spinal cord injury by western blot and Neun protein by immunofluorescence. Considering that the inflammatory microenvironment was altered prior to behavioral changes in the animals, to further investigate the mechanism of motor function recovery in rats, we examined brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor (GDNF) in the spinal cord tissues of rats on day 7. On day 7, expression levels of BDNF and GDNF proteins were significantly higher in LbGp-treated rat spinal cord tissues than those in the SCI group, as determined by western blotting (Fig.1B-D) (Fig.S3A-B). On day 7, the expression of Neun protein in spinal cord tissue was consistent with the above results, as determined in tissue immunofluorescence experiments (Fig.1E-F). Therefore, we suggest that LbGp could promote neuronal repair and improve motor function after SCI in rats by modulating BDNF and GDNF.

3.3 LbGp inhibit NF-kB and pyroptosis-related proteins in vivo

The role of LbGp in inhibiting neuroinflammation has been demonstrated[19]. To further confirm the role of LbGp in neuroinflammation, we administered different concentrations of LbGp after spinal cord injury by gavage treatment to assess the optimal concentration for its therapeutic effect. The inhibitory effect of different concentrations of LbGp on s neuroinflammation was assessed by western blot experiments. Rats were treated with different concentrations (10, 50, and 100 mg/kg) of LbGp after SCI, and the inflammatory factor Pro-IL-18 was detected in the different treatment groups. We observed that LbGP afforded superior inhibition of the inflammatory factory Pro-IL-18 50mg/kg (Fig.2A) (Fig.S3C). Accumulated evidence has shown that LBP can inhibit the expression of the NLRP3 inflammasome and ROS production to suppress inflammation [20]. To further elucidate the mechanism of LbGp's role in spinal cord injury-induced neuroinflammation, we used immunofluorescence and western blot to detect NLRP3, ASC, and Caspase-1 proteins. Compared to SCI group, expression levels of NLRP3, ASC, and Caspase-1 proteins determined by tissue immunofluorescence were significantly reduced in the LbGp group (Fig.2B-D). Based on the western blot analysis, rats in the SCI group exhibited elevated expression of P-p-65, NLRP3, ASC, Caspase-1p45, GSDMD, GSDMD-N, IL-1β and IL-18 proteins in the spinal cord at day 7 post-SCI; these expression levels were significantly reduced in the LbGp group (Fig.2E-M) (Fig.3D-E).

3.4 LbGp inhibit MAPKs/NF-kB and pyroptosis-related proteins in vitro

As mentioned above, LbGp can inhibit the NF-kB and pyroptosis pathways to suppress neuroinflammation in vivo, but the target cells on which it acts are unclear. Microglia are intrinsic immune cells in the CNS that are involved in the immune response in vivo and play an essential role in neuroinflammation[21]. To determine the effect of LbGp on microglia, a CCK-8 assay was used to examine the cell viabilities. The results indicated that there were no significant changes in cell viabilities when incubation in 100-400ug/ml LbGp (Fig.S4A). To further assess the optimal drug concentration for LbGp inhibition of inflammation triggered by microglia, we incubated LbGp at different concentrations (100,200
and 400ug/ml) for 24 h after microglia activation and assayed the expression levels of the inflammatory factor IL-18 mRNA. The RNA of IL-18 was reduced in both LbGp group (100,200 and 400ug/ml), and there was no significant difference between the LbGp groups (Fig.3A). Combining the above results, we gave 100ug/ml of LbGp intervention after microglia activation. As previously described, administration of LbGp treatment after activation of microglia resulted in lower expression of P-p38, P-JNK, P-p65, NLRP3, ASC, Caspase-1p20, GSDMD, GSDMD-N, IL-18, and IL-1β proteins in the LbGp-treated group compared to the ATP+LPS group (Fig.3B-K).

3.5 LbGp inhibits MAPKs/NF-kB and pyroptosis-related proteins by modulating DHA

As described before, LbGp inhibited neuroinflammation both in vivo and in vitro, and to further clarify the mechanism of LbGp treatment for spinal cord injury by metabolomic analysis, 66 metabolites were found to be elevated in cell supernatants and 38 in tissues in the LbGp-treated group (Fig.S5A-E). By matching comparisons, we found that both ethanolamine and docosahexaenoic acid were increased in tissue and cell supernatants after LbGp intervention (Fig.4C). At present, the role of ethanolamine in neuroinflammation is not clear [22-24]. The view that DHA suppresses inflammation by inhibiting the NLRP3 protein in activated macrophages is well confirmed. [25-27]. DHA intervention given after spinal cord injury in rats can inhibit neuroinflammation[28]. Therefore, we reasoned that LbGp may suppress neuroinflammation by regulating DHA production by microglia. To test this view, we administered SC-26196, a inhibitor of FADs2, a key enzyme for DHA production, before LbGp intervention, and the results showed that the effect of LbGp in inhibiting inflammation was significantly weakened after reduced DHA production (Fig.3B-L).

3.6 LbGp induces DHA secretion from microglia

Whether LbGp can induce DHA secretion from microglia, we detected cell supernatants by ELISA and found that DHA was significantly elevated in the LG intervention group (Fig.4D). Metabolomic analysis showed that microglia are rich in DHA, providing the possibility of DHA secretion[29]. FADS1 and FADS2 play an essential role in the process of DHA synthesis[30]. Q-PCR results showed that LbGp intervention significantly contributed to high expression of FADS1 and FADS2 mRNA, key enzymes for DHA production (Fig.4E-F).

3.7 DHA inhibit MAPKs/NF-kB and pyroptosis-related proteins in vitro

Previous study suggest that DHA can inhibit microglia-induced neuroinflammation[31]. Therefore, we further verify its role in the MAPKs/NF-kB and pyroptosis pathway through WB experiments. As previously described, administration of DHA treatment after activation of microglia resulted in lower expression of P-p38, P-JNK, P-p65, NLRP3, ASC, Caspase-1p20, GSDMD, GSDMD-N, IL-18, and IL-1β proteins in the DHA-treated group (Fig.A-K).
Discussion

In this study we demonstrated for the first time that oral LbGp can improve the SCI inflammatory microenvironment and promote spinal cord repair by inhibiting MAPKs-NF-κB/pyroptosis-related pathways, the exact mechanism of which has not been previously reported.

Spinal cord injury is often accompanied by hemorrhage, peripheral tissue edema, secondary neuroinflammation, and glial scar formation, and these adverse factors severely limit nerve regeneration and functional recovery[32, 33]. There are two stages of spinal cord injury; primary SCI includes spinal cord tissue breakdown, hemorrhage, and destruction of the glia membrane [32]. SCI is a delayed and progressive tissue injury following primary SCI. Ionic homeostasis imbalance in tissues after SCI leads to mitochondrial dysfunction and release of reactive oxygen species, resulting in oxidative stress damage to tissue; damaged cell lead to upregulation of excitatory amino acids and excitotoxicity leading to apoptotic necrosis; inflammatory cells such as microglia and macrophages infiltrate blood-brain-barrier disruption the damaged area and release inflammatory factors [34].

In recent years an increasing number of herbal extracts have been used to study their neuroprotective mechanisms of action. It has been reported that Gastrodia elata Blume could increase the cell viability of embryonic neural progenitor cells under hypoxic condition by improving DNA damage repair ability[35]. Danshen extract play a beneficial role in the recovery of locomotor function following SCI in rats. Meanwhile, this effect may be associated with the promotion of axonal regeneration, up-regulation of BDNF, and activation of microglial cells[36]. Iridoid glycosides of Paederia scandens possesses antinociceptive effect, which may be partly related to the inhibition of NO/cGMP/PKG signaling pathway in the rat spared nerve injury model of neuropathic pain[37]. Previous research revealed that aloe-emodin protected against brain damage, which is primarily attributed to the antioxidant and anti-neuroinflammatory properties of AE via the PI3K/AKT/mTOR and NF-kB activation[38]. LBP is an activity ingredient extracted from Lycium barbarum, which can improve inflammatory microenvironment [39, 40]. However, the mechanism of action of LBP derivative LbGp in spinal cord injury is unclear.

After spinal cord injury, a large amount of ROS and ATP can activate the release of NLRP3, ASC, Caspase-1, GSDMD and other key proteins related to pyroptosis and related inflammatory factors, which lay a very important role in spinal cord injury[11, 41]. To further verify the mechanism of action of LbGp in inhibiting pyroptosis in spinal cord injury, we found that the LbGp treatment groups could inhibit the expression of key protein p-P38/p-JNK in MAPKs pathways, p-P65 in NF-κB pathway, N LR3, ASC, caspase-1, GSDMD and downstream inflammatory factors such as IL-18 and IL-1β in western blot experiments. The specific mechanism of pyroptosis inhibition in the LbGp treatment groups has not been clarified. Herein, levels of unsaturated fatty acid DHA were elevated in the LbGp groups, as determined by assessing the metabolism of microglia and tissues. Previous studies have shown that the unsaturated fatty acid DHA can inhibit NLRP3 and other key proteins of pyroptosis[25]. Unsaturated fatty acids reduce injury-related oxidative stress, decrease microglia/macrophage responses, and improve motor function and bladder function recovery in rats [42]. Following entry into the brain, DHA is esterified into and recycled amongst membrane phospholipids contributing the distribution of DHA in brain phospholipids. During neurotransmission and following brain injury, DHA is released from membrane phospholipids and converted to bioactive mediators which regulate signaling pathways important to synaptogenesis, cell survival, and neuroinflammation, and may be relevant to treating neurological diseases[43]. It has been shown that 3 months of DHA treatment prevents microglia activation after ischemic injury, reduces the
size of ischemic lesions, and increases the level of the anti-apoptotic molecule Bcl-2 in the brain[44]. In addition, when we inhibit the DHA-producing pathway in microglia, the effect of LbGp to improve the neuroinflammation is reduced, suggesting that LbGp inhibits SCI pyroptosis by regulating unsaturated fatty acid DHA. Accordingly, LbGp contains diverse metabolites (Fig.S1A); however, which metabolite plays the main role in inducing DHA release from microglia is not clear and needs to be further elaborated in future experiments.

LbGp can promote BDNF and GDNF to repair nerve damage. GDNF is a potent promoter of central and peripheral neurons, and BDNF is a major regulator of energy homeostasis that upregulates antioxidant enzymes to enhance repair of damaged neurons, promote differentiation of neurons and stem cells, promote neural protrusion growth and synapse formation, and prevent programmed cell death and apoptosis[18, 45-48]. DHA can promote high BDNF expression [49]. We found that LbGp promoted high expression of BDNF and GDNF in vivo experiments with Western blot further validated this conclusion. The results of immunofluorescence protein NeuN in spinal cord tissue indicated that the LbGp treatment group promoted neuronal repair, and the results of behavioral BBB function score in rats were found that LbGp can improve motor function in rats after spinal cord injury. BBB motor function score suggests that treatment of spinal cord injury in rats with LbGp significantly improves motor function by the second week.

Therefore, we hypothesize that LbGp plays a role in the upregulation of BDNF and GDNF through modulate DHA in promoting the recovery of motor function in rats.

Conclusion
To the best of our knowledge, we, for the first time, report the mechanism of action of LbGp, a polysaccharide derivative of LBP, for treating SCI, which induce microglia secret DHA to inhibit cellular pyroptosis. Because LbGp is widely available at low cost and has an excellent safety profile, which will provide new direction for treating spinal cord injuries

Author Contribution
Zhanfeng Jiang, Zhong Zeng, He He, Mei Li, Pengfen Bie, Yuanxiang Lan, Jianwen Hui, and Yanjun Chen acquired experiment data, Hao Liu, afford material support, Zhanfeng Jiang drafted the manuscript, Hechun Xia and Heng Fan were involved in study design and supervision.

Conflict of interest
The authors declare that they have no conflict of interest.

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Abbreviation
ASC apoptosis-associated speck-like protein
References


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Lycium Barbarum Glycopeptide promote nerve regeneration and benefit motor function recovery. (A) Motor function was evaluated using the BBB score in each group, the LbGp treatment group had higher score than the SCI group since the second week and was not statistically different from the sham group. (n=3 for each group) *p 0.05, SCI VS LbGp treatment group. (B-D) detected the expression level of BDNF and GDNF in spinal cord tissue by western blotting. (E-F) Fluorescence staining micrographs of NEUN protein in spinal cord tissues of different groups. Data represent the means SEM of at least 3 independent experiments, Scale bar 200um. n=3 per group, *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001 versus each group.
Figure 2

Lycium Barbarum Glycopeptide inhibit NF-kB and pyroptosis-related proteins in vivo

(A) Spinal cord injury treatment with different doses (10mg/kg-100mg/kg) of LbGp of reduces pro-IL-18 protein expression levels. (B-C) Fluorescence staining micrographs of NLRP3, ASC, Caspase-1 protein 7th days in spinal cord tissues of different groups. (E-M) On day 7, the expression level of each group of
NLRP3, ASC, Caspase-1, p-P65, P-65, GSDMD, GSDMD-N, IL-18, IL-1β protein in spinal cord tissue was detected by western blotting. Data represent the means SEM of at least 3 independent experiments. Scale bar 200um, n=3 per group, *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001, versus each group.

Figure 3
Lycium Barbarum Glycopeptide inhibited microglia MAPKs/NF-kB and pyroptosis-related pathways. (A) mRNA expression level of IL-18 in ATP+LPS stimulated Microglia treatment with different doses (100ug/kg-400ug/ml) of LbGp. (B-L) The expression levels of p-P38, P38, p-JNK, JNK, p-P65, P-65, NLRP3, Caspase-1-p20, ASC, GSDMD, GSDMD-N , IL-18, IL-1β protein in each group after microglia were activated in western blotting experiments. Data represent the means SEM of at least 3 independent experiments *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001 versus each group.

Figure 4

Lycium Barbarum Glycopeptide Induces microglia to secrete DHA

(A) Heat map of differential metabolic substances between the SCI and LbGp groups in spinal cord tissue. (B) Heat map of differential metabolic substances between ATP+LPS and LbGp treatment groups in
microglia supernatants. (C) Venn diagram of elevated metabolites in spinal cord tissues LbGp-treated group and microglia LbGp-treated group. (D) After ATP+LPS activation of microglia for 4h, LbGp was given to incubate for 24h, cell supernatants were assayed for DHA. (E-F) mRNA expression level of FADs1 and FADs2 in ATP+LPS stimulated Microglia treatment with LbGp. Data represent the means±SEM of at least 3 independent experiments n=6 per group, *p<<0.05, **p<<0.01, ***p<<0.001, ****p<<0.0001 versus each group.

Figure.
Figure 5

DHA inhibited microglia MAPKs/NF-κB and pyroptosis-related pathways. (A-K) The expression levels of p-P38, p38, p-JNK, JNK, p-P65, P-65, NLRP3, Caspase-1-p20, ASC, GSDMD, GSDMD-N, IL-18, IL-1β protein in each group after microglia were activated in western blotting experiments. Data represent the means SEM of at least 3 independent experiments *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001 versus each group.
Figure 6

A model illustrating the role of LbGp in alleviating the inflammatory microenvironment by prompting microglia to secrete DHA to inhibit the MAPKs/NF-kB and pyroptosis pathway.

LbGP action on microglia promotes high expression of FADs1 and FADs2, key enzymes for GD synthesis on the endoplasmic reticulum, prompting microglia to secrete DHA, which further and improves the inflammatory microenvironment via inhibits MAPKs/NF-kB and pyroptosis pathway. However, the intervention of the FADs2 inhibitor SC-26196 diminishes the inhibitory effect of LbGp on neuroinflammation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• Supplementmaterial.pdf
• Table.docx