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Lycium Barbarum Polysaccharides Upregulate Trx2 in Schwann Cells through ESR1 to Repair Sciatic Nerve Injury in Rats

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ABSTRACT: Objectives: Sciatic nerve injury (SNI) impairs quality of life, and *Lycium barbarum polysaccharides* (LBP) may exert therapeutic effects via regulating Schwann cell (SC) mitochondrial stability, though the mechanism remains unclear. The study aimed to elucidate the therapeutic mechanisms of LBP in mitigating sciatic nerve injury by protecting Schwann cells via the estrogen receptor 1 (ESR1)/thioredoxin 2 (Trx2) pathway. **Methods:** An *in vitro* SNI model was established by inducing RSC96 cells with H₂O₂. Cell counting kit-8 (CCK8) assay, enzyme-linked immunosorbent assay (ELISA), Western blot, reactive oxygen species (ROS) and adenosine triphosphate (ATP) quantification, and mitochondrial membrane potential (MMP) detection were used to evaluate cellular functions and molecular changes. Network pharmacology and molecular docking were employed to predict potential targets of LBP. Chromatin immunoprecipitation (ChIP) assays verified the interaction between ESR1 and Trx2, and ESR1 was knocked down in SCs to confirm the regulatory pathway. Additionally, *in vivo* validation was performed using a rat SNI model. **Results:** LBP intervention significantly reduced H₂O₂-induced mitochondrial dysfunction, inflammation, and oxidative stress in RSC96 cells ($p < 0.05$). It also regulated the expression of apoptosis-related proteins, Trx2, and mitochondrial apoptosis-inducing factor (AIF; $p < 0.001$). Network pharmacology and molecular docking identified ESR1 as a key target of LBP, and ChIP assays confirmed that ESR1 promoted Trx2 transcription. Moreover, LBP enhanced SC proliferation (OD₄₅₀ at day 5 = 1.25-fold; colony formation = 1.85-fold), increased MMP and ATP levels (MMP = 2.25-fold; ATP = 1.75-fold), inhibited inflammation, and reduced apoptosis ($p < 0.001$), while ESR1 knockdown abrogated these effects ($p < 0.05$). In rat SNI models, LBP effectively alleviated nerve injury ($p < 0.001$). **Conclusion:** LBP may modulate Trx2 via ESR1 for the purpose of restoring the dysfunction of SCs induced by H₂O₂. This potentially offers a new strategy for the therapy of SNI.

KEYWORDS: *Lycium barbarum* polysaccharide (LBP); sciatic nerve injury (SNI); Schwann cells (SCs); estrogen receptor 1 (ESR1); thioredoxin 2 (Trx2); mitochondrial membrane potential (MMP)

1 Introduction

Etiological factors ranging from hip dislocation and sharp trauma to pharmaceutical injections are recognized as the primary precipitants of sciatic nerve injury (SNI), a frequent manifestation of peripheral nerve injury (PNI) [1,2]. The consequent physiological deficits, specifically the cessation of lower limb sensory and motor function coupled with muscular paralysis and atrophy, are identified as the critical determinants driving the severe compromise in patient quality of life. At present, the surgical treatment of SNI mainly relies on neurolysis, nerve anastomosis, and nerve implantation. Furthermore, the reinstatement of neurological integrity is attainable via neurotrophic agents; notably, the attenuation of inflammatory

cascades and edema is mediated by therapeutics such as glucocorticoids and non-steroidal anti-inflammatory drugs [3,4]. Unfortunately, the therapeutic effects of the above methods are still not satisfactory. Research reports have shown that stem cells and gene therapy have also shown great potential in treating SNI by expressing specific neurotrophin [5,6], the high cost and the extraction and culture of autologous cells limit their further development. Therefore, it is extremely important to explore new therapeutic strategies for SNI.

Schwann cells (SCs) have a complex role in the peripheral nervous system, such as conducting electrical signals, providing axonal nutrition, metabolic support, and mediating the repair and regeneration in injured nerves through Wallerian degeneration [7]. These functions rely on mitochondria to provide energy and significantly affect the pathological progression of the SNI. Therefore, SCs are in urgent need of maintaining mitochondrial homeostasis. Nevertheless, the exacerbation of cellular homeostasis perturbation is driven by the post-traumatic hyperactivity of SCs, a mechanism that ultimately precipitates mitochondrial dysfunction in conjunction with the supraphysiological accrual of reactive oxygen species (ROS) [8,9]. In addition, some studies reported that the morphological changes of SCs in neuropathy were mainly focused on mitochondria [10,11], which made scholars realize that mitochondrial stabilization is essential for the function of SCs. Thus, restoring mitochondrial homeostasis after injury can help reduce the intracellular ROS pool accumulation and promote the healing of SNI.

Lycium barbarum (LB) originated in China, is suitable for both medicine and food nutrition, and is a health-care vegetable and traditional Chinese medicine [12]. The derivation of *Lycium barbarum* polysaccharides (LBP), classified as a bioactive moiety, is achieved through extraction and purification processes applied to LB [13]. Its antioxidant, anti-inflammatory, and other functions can prevent and improve a variety of neurological diseases [14]. The prevention of optic neuron degeneration and the preservation of retinal ganglion cell integrity alongside retinal functionality were demonstrated in a murine model of ocular hypertension through LBP intervention [15]. Moreover, the amelioration of CORT-induced cognitive deficits was attributed to the LBP-mediated regulation of CRHR1 expression [16]. However, the role of LBP in SNI and mitochondrial homeostasis of SCs remains unclear.

The primary objective of the current investigation was the delineation of the specific involvement of LBP within the SNI context, concurrent with an interrogation regarding its capacity to modulate the phenotypic characteristics and functional integrity of Schwann cells. It is considered to be relatively important to clarify the specific molecular mechanisms of LBP in the process of regulating mitochondrial dysfunction and the levels of reactive oxygen species (ROS).

2 Materials and Methods

2.1 Cell Lines

The specific RSC96 rat Schwann cell line (SCs) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA; CRL-2765). For the purpose of facilitating cell growth, a culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM; PM150210A, Procell, Wuhan, China) supplemented with approximately 10% fetal bovine serum (FBS; SH31194.07, Cytiva, Shanghai, China) was used. In addition, penicillin (100 U/mL) and streptomycin (100 µg/mL) were included in the solution as relevant antimicrobial agents. The atmospheric conditions during the incubation process were generally maintained at a temperature of approximately 37°C with a CO₂ concentration of about 5%. Furthermore, the cells were analyzed using short tandem repeat genotyping for the purpose of identification, and the results of the mycoplasma test were found to be negative.

Sterile physiological saline was generally used as the solvent for the purpose of dissolving LBP ($\geq 95\%$ purity, LBP1291, BSZH, Beijing, China). To determine the effect of LBP on H_2O_2 -treated SCs, SCs were pretreated with 1 mM H_2O_2 (H112517, Aladdin, Shanghai, China) for 30 min before the treatment with different concentrations of LBP (0, 10, 50, 100, 200, 400, and 800 $\mu\text{g/mL}$) for 24 h.

2.2 Cell Viability and Proliferation Assay

RSC96 cells were generally observed to be in the logarithmic growth phase following the process of lentiviral infection. These cells were then distributed into relevant 96-well plates at a density of approximately 5000 cells per well, which was done in accordance with the specific experimental arrangement. After the plates were allowed to stand for a relatively short period, the chambers were examined under a microscope (TS2R, Nikon, Tokyo, Japan) to check for uniformity. Subsequently, the plates were placed in an incubator for the purpose of further cultivation. The collection of absorbance data at 450 nm was carried out using a microplate reader (SpectraMax, Molecular Devices, San Jose, CA, USA) at various time points (1, 2, 3, 4, and 5 days) or at approximately 24 h after the introduction of 0, 10, 50, 100, 200, 400 and 800 $\mu\text{g/mL}$ LBP ($\geq 95\%$ purity, LBP1291, BSZH, Beijing, China). Prior to this measurement, the cells were subjected to an incubation period of about 2 to 4 h. This step was performed following the addition of 10 μL of the CCK-8 detection reagent (P-CA-001, Procell, Wuhan, China).

For the colony formation assay, RSC96 cells from different treatment groups were seeded in 6-well plates at 1×10^3 cells per well and cultured to allow colony formation. After 14 days of incubation, colonies were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) and stained with crystal violet staining solution (IC0600, Solarbio, Beijing, China). The number of colonies was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.3 ROS Detection Assay

The detection of ROS levels in RSC96 cells was performed using the ROS Assay Kit (S0033S, Beyotime, Shanghai, China) in strict accordance with the manufacturer's recommended protocol. In brief, the DCFH-DA probe was diluted 1:1000 in Phosphate-Buffered Saline (PBS) to a final concentration of 10 $\mu\text{mol/L}$. Harvested cells were resuspended in the diluted probe at 1×10^6 cells/mL and incubated at 37°C for 20 min. Cells were washed three times with PBS to remove unincorporated DCFH-DA, then aliquoted for stimulation. ROS levels were measured via a microplate reader (SpectraMax, Molecular Devices, San Jose, CA, USA), with excitation at 488 nm and emission at 525 nm.

2.4 Mitochondrial Membrane Potential Analysis

The assessment of potential variations regarding the mitochondrial membrane potential (MMP) in RSC96 cells was generally conducted in accordance with the specific protocols provided by the manufacturer, using a detection kit (C2006, Beyotime, Shanghai, China) designed for this purpose. The experimental workflow necessitated the initial evacuation of culture medium and duplicate $1 \times \text{PBS}$ (pH 7.4) rinsing, prior to a 10 min fixation phase mediated by 4% paraformaldehyde. Labeling was thereafter accomplished by the integration of 1 mL culture fluid with an equivalent volume of 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) working solution, incubated at 37°C for 20 min. Final analysis via fluorescence microscopy (BX53, Olympus, Tokyo, Japan) was conducted subsequent to the removal of unbound dye through a JC-1 buffer wash. Red fluorescence indicates aggregation of JC-1 dye but green indicates mitochondrial dysfunction.

2.5 Adenosine Triphosphate (ATP) Production Assay

Cellular ATP levels were measured using a commercial ATP assay kit (ab83355, Abcam, Cambridge, UK) following the manufacturer's guidelines. Briefly, RSC96 cells were lysed, and the lysates were centrifuged at $12,000\times g$ for 5 min. Twenty microliters of the supernatant was mixed with 100 μL ATP detection working solution, and the luminescence intensity was immediately determined using a luminometer (Luminoskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA).

2.6 Enzyme-Linked Immunosorbent Assay (ELISA) Assay

The levels of tumor necrosis factor- α (TNF- α ; PT516, Beyotime, Shanghai, China), interleukin-6 (IL-6; PI328, Beyotime, Shanghai, China), and IL-1 β (PI303, Beyotime, Shanghai, China) were quantified by ELISA in strict accordance with the manufacturer's instructions. Briefly, 100 μL of cell supernatant samples were mixed with the sample assay buffer. Next, 96-well plates were sealed and incubated at room temperature (RT) for 120 min. After five washes, 100 μL pre-prepared biotinylated TNF- α /IL-6/IL-1 β antibody was added, sealed and incubated at RT for 60 min. Plates were washed five times again, then 100 μL HRP-conjugated streptavidin was added, sealed with an opaque membrane, and incubated at RT for 20 min in the dark. Following five washes, 100 μL TMB substrate was added, sealed, and incubated at RT for 20 min in the dark. The reaction was terminated with 50 μL stop solution per well, and the absorbance at 450 nm was immediately measured.

2.7 Target Prediction and Molecular Docking

The chemical constituents and target data of LBP were from the Traditional Chinese Medicines Integrated Database (TCMID) (<https://www.bidd.group/TCMID/>). The chemical constituents of LBP were searched by using the keyword "*Lycium barbarum* polysaccharides". The structure data files (SDF) of the above compounds were obtained by using the database export function. All compound structures were initially retrieved as 2D formats from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and were subsequently converted to 3D structures through energy minimization using the MMFF94 force field in Open Babel (version 3.1.1). Compound validation was grounded in the PubChem database. A filtration protocol was subsequently implemented via the Swiss Target Prediction engine (<http://swisstargetprediction.ch/>, version 2019.02), confining selection to targets displaying a prediction score > 0 . In parallel, the GeneCards database (<https://www.genecards.org/>, version 5.13) served as the source for SNI-associated pathological targets. The convergence of these predicted and disease-oriented datasets, post-redundancy elimination, facilitated the isolation of potential LBP therapeutic targets. The intersection of these predicted and disease-oriented datasets was identified using a Venn diagram analysis (<https://bioinfo.gp.cnb.csic.es/tools/venny/index.html>) to screen for common molecular targets shared between LBP and the disease state. The three-dimensional structure of the target protein (PDB ID: 1A52) was obtained from the RCSB PDB database (<https://www.rcsb.org/>). Protein preparation included removal of water molecules and original ligands, addition of hydrogen atoms, assignment of Gasteiger charges, and definition of the binding pocket using the coordinates of the native ligand. The grid box for docking was centered at coordinates (X, Y, Z) with dimensions (A, B, C) Å, covering the predicted active site. Molecular docking and mapping were carried out by using software AutoDock (version 4.2.6) and PyMOL (version 2.5), and the binding stability between components and proteins was judged according to the binding energy of small molecule compounds and protein docking.

2.8 Assessment of Protein-Protein Interaction Network Topologies and Functional Pathway Enrichment

The derivation of the protein-protein interaction (PPI) network was achieved through the importation of potential targets into the STRING database (<https://cn.string-db.org/>), imposing a minimum confidence threshold of 0.4. Following data acquisition, the intersecting targets were subjected to computational analysis within the R environment (version 4.2.1), specifically involving the execution of Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathway assessments.

2.9 Real-Time Fluorescent Quantitative PCR

Total cellular RNA was isolated using Trizol reagent (R0016, Beyotime Biotechnology, Shanghai, China). Briefly, harvested cells washed with 1× PBS (pH 7.4) were lysed with 1 mL of Trizol. After homogenization, 200 µL of chloroform was added, and the mixture was vortexed thoroughly and incubated at room temperature for 5 min. The sample was then centrifuged at 12,000× g for 15 min at 4°C using a Centrifuge 5430 R (Eppendorf SE, Hamburg, Germany). Following centrifugation, the upper colorless aqueous phase containing RNA was carefully transferred to a new tube. RNA was precipitated by adding an equal volume of isopropanol, incubating at −20°C for 30 min, and centrifuging at 12,000× g for 15 min at 4°C. After this step, the supernatant was discarded, and the RNA pellet was washed once with 75% ethanol (centrifugation at 7500× g for 5 min at 4°C). The pellet was air-dried for 5–10 min and finally resuspended in RNase-free water. RNA concentration and purity were assessed using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA with an A260/A280 ratio between 1.8 and 2.0 was used for subsequent steps. A total of 1 µg of RNA from each sample was reverse-transcribed into cDNA using the Hiscript QRT Supermix kit (R223-01, Vazyme, Nanjing, China) according to the manufacturer's protocol. qRT-PCR amplification was performed using SYBR Green master mix (MonAmp™, Monad, Suzhou, China) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Cycle threshold (Ct) values were recorded, and the relative expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH serving as the reference gene for normalization. The primers used for Reverse Transcription Quantitative PCR (qRT-PCR) are listed in Table 1.

Table 1: The primers for qRT-PCR and chromatin immunoprecipitation (ChIP).

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Trx2-ESR1-1	TAGAAGTACCGCCAGCCCT	GCATTAAGGGGTATTCTGCCGT
Trx2-ESR1-2	AGAACAGAGCTTCCTCGGCAT	CTTTGGAACAAGCTCCTGCTGT
Trx2-ESR1-3	AGAGACGTTGTCCATGTGTTCTG	CTGTCCACCTTCCCTTCCATACC
ESR1	CTACTGAACGCGGTGACAGA	CGTGTGAGCATTCAGCATCT
TXN2	AAGCCTCCTCAAGGTGTGTG	CTGGTGGTGTGAAATGTCCG
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA

2.10 Chromatin Immunoprecipitation (ChIP) Assay

ChIP analysis was performed using the EpiQuik ChIP Kit (P-2002-1, Epigentek, Farmingdale, NY, USA) in strict compliance with the manufacturer's instructions. Briefly, cells were first cross-linked with 1% formaldehyde to stabilize protein-DNA interactions, followed by glycine quenching to terminate the reaction. Cells were then lysed, and chromatin was fragmented to 200–500 bp via MNase digestion or sonication. After reserving input control for normalization, chromatin was incubated overnight at 4°C with

anti-estrogen receptor 1 (ESR1) antibody (ab32063, 1:100, abcam, Cambridge, UK) or IgG (ab172730, 1:100, abcam, Cambridge, UK). Antibody-protein-DNA complexes were captured by agarose beads, extensively washed to remove impurities, eluted, and cross-links reversed. DNA was purified after proteinase K digestion to degrade proteins, and finally analyzed by qPCR to detect target DNA enrichment.

2.11 Design of shRNA, Overexpression Plasmids, and Lentiviral Transfection

The distinct short hairpin RNA (shRNA) sequences targeting *ESR1* (NM_000125.4) were designed using an online algorithm. The sequences were as follows: shESR1-1, 5'-TGATCAAAACGCTCTAAGAA-3'; shESR1-2, 5'-GTGTGCCTCAAATCTATTATT-3'; shESR1-3, 5'-CTACAGGCCAAATTCAGATAA-3'. The corresponding single-stranded DNA oligonucleotides (synthesized by Tsingke Biotechnology, Beijing, China) were annealed to form double-stranded DNA inserts. These inserts were then cloned into the linearized pLKO.1-puro vector (Addgene, Watertown, MA, USA) between the AgeI and EcoRI restriction sites using T4 DNA ligase. The constructed plasmid was transformed into competent *E. coli* cells. Positive clones were verified by Sanger sequencing (Sangon Biotech, Shanghai, China), and the correct plasmid was extracted in large quantities using an EndoFree Maxi Plasmid Kit (Tiangen, Beijing, China).

For lentiviral production, the shRNA plasmid (or a corresponding *ESR1* overexpression plasmid) was co-transfected with the packaging plasmids psPAX2 and the envelope plasmid pMD2.G (both from Addgene) into cells using Lipofectamine™ 3000 (Thermo Fisher Scientific, Carlsbad, CA, USA). The medium was replaced 6 h post-transfection. Viral supernatants were collected at 48 and 72 h, filtered through a 0.45 µm membrane, and concentrated by ultracentrifugation.

The titer of the lentiviral particles was determined by transducing HEK293T cells with serial dilutions of the viral stock and counting puromycin-resistant colonies. RSC96 cells were then transduced with lentivirus at a multiplicity of infection (MOI) of 20 (corresponding to $\sim 1 \times 10^8$ TU/mL) in the presence of 8 µg/mL Polybrene. Transduction efficiency was estimated 72 h later by observing the fluorescence of a parallel control virus encoding GFP. Stable cell lines were selected and maintained in medium containing 2 µg/mL puromycin for one week. Confirmation of successful gene knockdown or overexpression in the established stable cell lines was secured via the execution of qRT-PCR and Western Blot analyses.

2.12 Western Blot Assay

Western blot analysis was performed to examine the transfection efficiency of *ESR1* and the expression levels of apoptosis-related proteins. First, cells were lysed on ice for 30 min using RIPA Lysis Buffer (P0013B, Beyotime Biotechnology, Shanghai, China), and then the lysate was centrifuged at $12,000 \times g$ for 15 min at 4°C to collect the supernatant. Following lysis and centrifugation, protein concentrations were determined using a BCA kit (P0012, Beyotime, Shanghai, China). 30 µg proteins were separated by SDS-PAGE (P0012A, Beyotime, Shanghai, China), transferred to PVDF membranes (FFP19, Beyotime, Shanghai, China), and blocked with 5% skim milk in 1× TBST (1 h). The membranes were incubated overnight at 4°C with the following primary antibodies listed in Table 2. After washing, membranes were incubated with HRP-conjugated secondary antibodies (P1311S, Beyotime, Shanghai, China). Protein bands were visualized using an ECL chemiluminescent kit, and band intensities were quantified with Image J software (National Institutes of Health, Bethesda, MD, USA). The relative expression level of each target protein was calculated by normalizing its gray value to that of the internal control GAPDH.

Table 2: Western blot antibodies information.

Antibody	kDa	Dilution Ratio	Species	Manufacturer	Catalog Number
ESR1	70	1:500–1:2000	Rabbit	Proteintech	21244-1-AP
Caspase 3	35	1:500–1:2000	Rabbit	Proteintech	19677-1-AP
BAX	20	1:2000–1:16,000	Rabbit	Proteintech	50599-2-Ig
Bcl2	25	1:2000–1:10,000	Rabbit	Proteintech	12789-1-AP
Trx2	15	1:500–1:2000	Rabbit	Proteintech	13089-1-AP
AIF	70	1:1000–1:8000	Rabbit	Proteintech	17984-1-AP
VDAC1	25	1:1000–1:6000	Rabbit	Proteintech	55259-1-AP
GAPDH	35	1:30,000	Mouse	Proteintech	60004-1-Ig
Goat Anti-Rabbit	/	1:3000	Goat	Beyotime	A0208
Goat Anti-Mouse	/	1:3000	Goat	Beyotime	A0216

2.13 Animals and Grouping

Adult male Sprague-Dawley rats (220–250 g) were sourced from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were generally housed under environmental conditions that were kept relatively standard. These conditions typically included a light/dark cycle of approximately 12 h, an ambient temperature of about $22 \pm 1^\circ\text{C}$, and a humidity level ranging from 50% to 60%. In addition, unrestricted access to food and water was provided for the animals throughout the process. The care and use of the laboratory animals were generally carried out in accordance with the NIH Guidelines. All animal experiments were conducted in accordance with the ARRIVE guidelines, and a detailed experimental flowchart was prepared accordingly (Fig. S1). This was done following the receipt of ethical approval from the Institutional Animal Care and Use Committee of the People's Hospital of Ningxia Hui Autonomous Region (Approval No: [2023]-NZR-103). Protocols for randomization were used to divide the subjects into three separate experimental groups ($n = 5$). These groups consisted of the Sham group, where surgical exposure was performed without damaging the nerve, the SNI model group, which involved the induction of injury to the sciatic nerve, and the Model + LBP group, where the SNI induction was combined with the administration of LBP.

2.14 Sciatic Nerve Injury (SNI) Model Surgery

The SNI model was generally carried out in accordance with protocols that had been previously established [17]. Under the condition of anesthesia induced by sodium pentobarbital (approximately 50 mg/kg, i.p. [18]), the right sciatic nerve and its three terminal branches, specifically the tibial, common peroneal, and sural nerves, were exposed through an incision made in the gluteal region. In the process of the surgery, the sural nerve was typically left intact. However, the tibial and common peroneal nerves were subjected to tight ligation using 5-0 silk sutures. This was usually followed by the removal of a distal segment measuring approximately 2 to 4 mm. In contrast, the animals in the sham group were subjected to exposure of the sciatic nerve only, and this was done without performing any nerve ligation or transection.

2.15 Drug Administration

Sterile physiological saline was generally used as the solvent for the purpose of dissolving Lycium barbarum polysaccharide (LBP, $\geq 95\%$ purity, LBP1291, BSZH, Beijing, China). A protocol for daily intragastric delivery (approximately 50 mg/kg) was established for the Model + LBP group, which typically covered the period from the day immediately following the operation until the end of the experiment (8 weeks). At the

same time, the administration of a relatively equal volume of saline vehicle was carried out for the Sham and SNI model groups, and this was generally done according to a similar time schedule.

2.16 Sciatic Functional Index (SFI) Assessment

The recovery of functional motor skills was generally measured using the analysis of the Sciatic Functional Index (SFI) [19], which was typically conducted at the time points of approximately 4 and 8 weeks after the operation. The recording of walking patterns was usually carried out by applying ink to the surfaces of the hind paws. This process allowed for the footprints to be recorded as the animals walked along a corridor lined with paper (measuring about 8.2 cm × 42 cm). The measurements were generally focused on three main spatial parameters: Print Length (PL), which is the distance from the heel to the third toe; Toe Spread (TS), which is the width between the first and fifth toes; and Intermediate Toe Spread (ITS), which is the distance between the second and fourth toes. The final determination of the SFI was typically based on the use of these values, which were obtained from both the experimental (E) and the healthy (N) limbs on the opposite side. These values were then processed according to the equation shown below:

$$SFI = [-38.3 \times (EPL - NPL) / NPL] + [109.5 \times (ETS - NTS) / NTS] + [13.3 \times (EIT - NIT) / NIT] - 8.55$$

In general, the integrity of function seems to be represented by an SFI value that is approaching 0. On the other hand, a score of approximately -100 is typically thought to suggest a state of relatively complete impairment.

2.17 Histological Analysis

Euthanasia was generally carried out via an overdose of sodium pentobarbital (100 mg/kg) at intervals of approximately 4 and 8 weeks after the surgery. Following the removal of the sciatic nerve segments located distal to the specific injury site, the tissue was typically subjected to fixation in 4% paraformaldehyde for a period of about 24 h. The subsequent processing steps generally included dehydration and embedding in paraffin at 58–60°C. This process eventually led to the preparation of transverse sections that were approximately 5 µm in thickness. The analysis of morphology was usually conducted using standard Hematoxylin and Eosin (H&E) staining (C0105S, Beyotime, Shanghai, China). At the same time, the assessment of tissue necrosis was carried out on adjacent sections through the use of 1% Toluidine Blue (HY-D0220, MedChemExpress, Shanghai, China). The capturing of images and the examination under a microscope were typically performed using a Nikon Eclipse Ci system (Nikon, Tokyo, Japan), and this was done under blinded conditions to a certain extent.

2.18 Statistical Analysis

For *in vitro* experiments, RSC96 cells were randomly assigned to different treatment groups using a random number table. For *in vivo* experiments, rats were randomly divided into three groups using a random number table. In addition, experiment operators and data analysts were unaware of group assignments during cell or animal handling, sample testing, and statistical analysis, and pathological evaluations were conducted in a blinded manner to ensure result objectivity. The reproducibility of the results was generally verified by performing all of the experimental assays in triplicate. The analysis of the statistical data was typically carried out using the GraphPad Prism software (GraphPad Software 10.1, San Diego, CA, USA). In general, the results are presented as the mean ± standard deviation (SD). Two-tailed Student's *t*-tests and one-way analysis of variance (ANOVA) with Tukey's post-hoc test were used for comparisons between two

groups; Fisher's exact or chi-square tests were applied for categorical data. A *p*-value threshold of less than 0.05 was typically considered to indicate statistical significance to a certain extent.

3 Results

3.1 LBP Decreases H₂O₂-Induced ROS Level Rise and Mitochondrial Membrane Potential Depolarization

The measurement of post-treatment survival was generally achieved through the use of cell viability assays, which were conducted across a range of different LBP concentrations. The analysis of the data seemed to reveal that, when compared to the control group, the application of relatively low doses of LBP (10 µg/mL and 50 µg/mL) did not appear to cause any statistically significant changes in the viability of the RSC96 cells (Fig. 1A). On the other hand, when the concentration of LBP was increased, the viability of the rat SCs RSC96 appeared to show a certain level of toxicity that was dependent on the concentration. It was observed that the cell survival rate was approximately 90% when the concentration of LBP was around 400 µg/mL. In addition, this rate was about 74% when the concentration was further increased to 800 µg/mL (Fig. 1A). This seems to suggest that the LBP concentration might not be fully supportive of cell survival at these levels. The findings seemed to indicate that therapy using LBP might have relatively good compatibility with SCs. Therefore, a dosage of approximately 400 µg/mL of LBP was selected for the purpose of the subsequent *in vitro* analysis. The evaluation of mitochondrial integrity generally requires the simultaneous assessment of the generation of ROS and MMP. A statistically significant reduction in the levels of ROS appeared to be evident in the LBP + H₂O₂ group relative to the H₂O₂ controls. This observation seemed to support the potential antioxidant and anti-inflammatory capabilities of LBP in rat SCs (Fig. 1B). In addition, when compared with the control group, the treatment with H₂O₂ appeared to block the normal function of the mitochondria. This seemingly resulted in a significant decrease in the production of MMP (Fig. 1C). Despite the apparent lack of a statistically significant difference in MMP between the LBP and the control groups, the potential therapeutic use of LBP in helping to improve mitochondrial dysfunction induced by H₂O₂ within RSC96 cells seemed to be supported to a certain extent. In the meantime, when compared with the control group, the treatment with H₂O₂ appeared to block the normal function of the mitochondria. This seemingly resulted in a significant decrease in the production of ATP and MMP (Fig. 1C,D). Despite the apparent lack of statistically significant differences in ATP and MMP between the LBP and the control groups, the potential therapeutic use of LBP in helping to improve mitochondrial dysfunction induced by H₂O₂ within RSC96 cells seemed to be supported to a certain extent. In addition, inflammatory responses were evaluated via cytokine detection, and it was found that H₂O₂ sharply elevated TNF-α, IL-6, and IL-β levels, while LBP co-treatment significantly suppressed these pro-inflammatory cytokine secretions (Fig. 1E–G). Collectively, LBP protects RSC96 cells from H₂O₂-induced oxidative damage, mitochondrial impairment, and inflammatory activation.

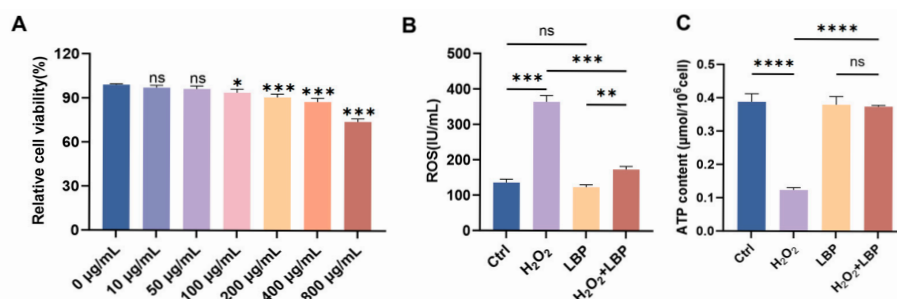


Figure 1: Cont.

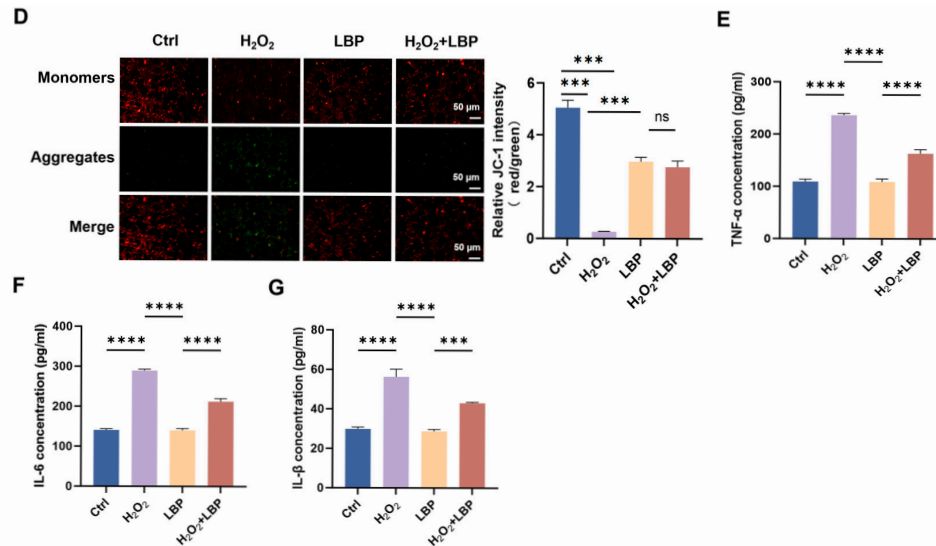


Figure 1: LBP alleviates H₂O₂-induced ROS burst, mitochondrial dysfunction and inflammation activation. (A) Effects of different concentrations of LBP on the viability of RSC96 cells. (B) The quantification of ROS intensity in each group (n = 3 independent biological replicate experiments). (C) The quantification of ATP content in each group (n = 3 independent biological replicate experiments). (D) Representative images of JC-1 staining in different groups and quantification of red/green fluorescence intensity (n = 3 independent biological replicate experiments). Scale bar = 50 μm. (E–G) The concentrations of pro-inflammatory cytokines TNF-α (E), IL-6 (F), and IL-1β (G) in different groups (n = 3 independent biological replicate experiments). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001; ns, no significance. Abbreviations: LBP, *Lycium barbarum* polysaccharides; ROS, reactive oxygen species; ATP, adenosine triphosphate; JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

3.2 LBP Promotes the Expression of Anti-Apoptotic Proteins and Inhibits Apoptosis

It is generally understood that a decline in MMP is often considered to be a marker of early apoptosis to a certain extent. Apoptosis-inducing factor (AIF) is typically found in the mitochondrial flavin protein. This protein not only helps to maintain mitochondrial energy metabolism, but it also appears to be a key effector molecule that may trigger apoptosis in Caspase pathways. Given the important role of thioredoxin 2 (Trx2) as a mitochondrial antioxidant in the process of maintaining redox homeostasis, energy metabolism, and the regulation of apoptosis, mitochondrial protein fractions were isolated. This was done for the purpose of examining the changes in AIF and Trx2 following the administration of LBP. The results of the analysis seemed to show that, when compared to the H₂O₂ challenge group, treatment with LBP appeared to induce a statistically significant reduction in the expression of AIF. This was also accompanied by an increase in the levels of mitochondrial Trx2 (Fig. 2A). This suggests that LBP not only contributes to reversing SCs' apoptosis levels in the injury model *in vitro*, but also effectively restores mitochondrial redox homeostasis. More importantly, only LBP treatment was able to effectively reduce mitochondrial apoptosis-related protein and maintain its stability (Fig. 2A). In addition, the changes of Caspase3, Bcl2, BAX, and Trx2 proteins were detected to evaluate the level of apoptosis in the cytoplasm. LBP treatment markedly suppressed the expression of the proapoptotic markers Caspase-3 and BAX, whilst simultaneously enhancing the levels of the antiapoptotic proteins Bcl-2 and Trx2 (Fig. 2B).

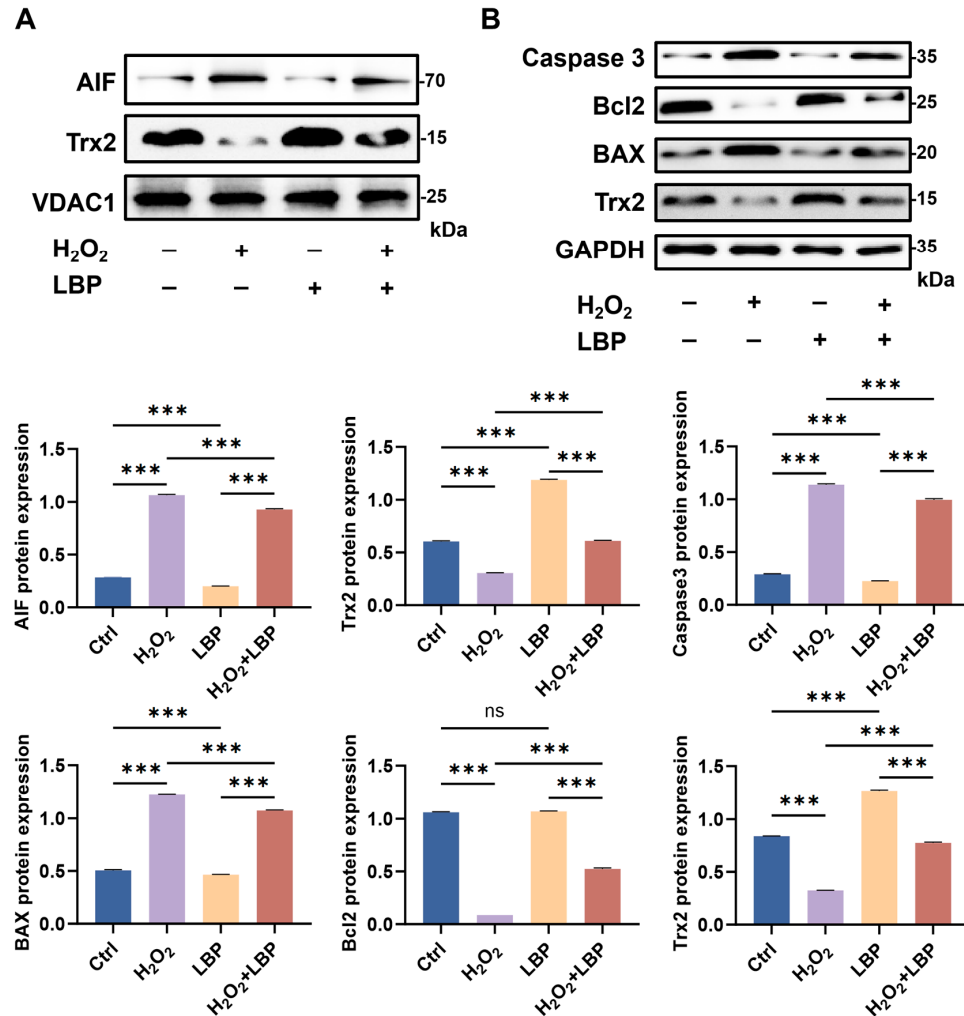


Figure 2: LBP increases apoptosis resistance and antioxidant capacity in RSC96 cells. (A) Representative Western Blot images of mitochondrial AIF and Trx2 protein changes and quantification in different groups (n = 3 independent biological replicate experiments). (B) Representative Western Blot images of cell apoptosis-related protein caspase3, Bcl2, BAX, and Trx2 protein changes and quantification in different groups (n = 3 independent biological replicate experiments). ****p* < 0.001; ns, no significance. Abbreviations: LBP, *Lycium barbarum* polysaccharides; AIF, apoptosis-inducing factor.

3.3 LBP Regulates Trx2 Expression by Transcription Factor ESR1

We further explored the target of LBP and the downstream molecular mechanism of mitochondrial apoptosis and redox homeostasis. After obtaining the chemical composition of LBP and its SDF, the potential targets were predicted by the STRING database and GeneCards database. As shown in the results, 57 genes in the intersection of the 584 predicted targets for LBP and 297 predicted targets for SNI were significantly correlated (Fig. 3A). The protein-protein interaction (PPI) network for the identified targets was mapped utilizing the STRING database, revealing 526 interactions involving 57 genes (Fig. 3B). Further exploration using KEGG pathway enrichment analysis in 57 genes showed a strong correlation between LBP with the neurological associative Alzheimer's disease, IL-17, and TNF inflammation-related signaling (Fig. S2). LBP may have a significant effect on the cell membrane raft and membrane microdomain by GO functional enrichment analysis (Fig. S3). In addition, by correlating 57 potential targets with

Trx2-predicted transcription factors, we found significant associations with the ESR1, androgen receptor (AR), and proto-oncogene (JUN). Among them, ESR1 showed the most stable binding force with LBP (Fig. 3C). The ChIP results confirmed that LBP could enhance the binding of ESR1 to the Trx2 promoter (Fig. 3D). Next, three shRNAs targeting ESR1 (shESR1-1/2/3) were transfected into RSC96 cells. Compared with the control group, shESR1-1, shESR1-2, and shESR1-3 all significantly downregulated the mRNA level of ESR1, and shESR1-3 was selected for subsequent analysis due to its strongest inhibitory effect (Fig. 3E). The following qRT-PCR results showed that the knockdown of transcription factor ESR1 greatly blocked the upregulation of ESR1 and Trx2 genes by LBP (Fig. 3F,G). Therefore, we subsequently analyzed the relationship between LBP and ESR1 in RSC96 cells.

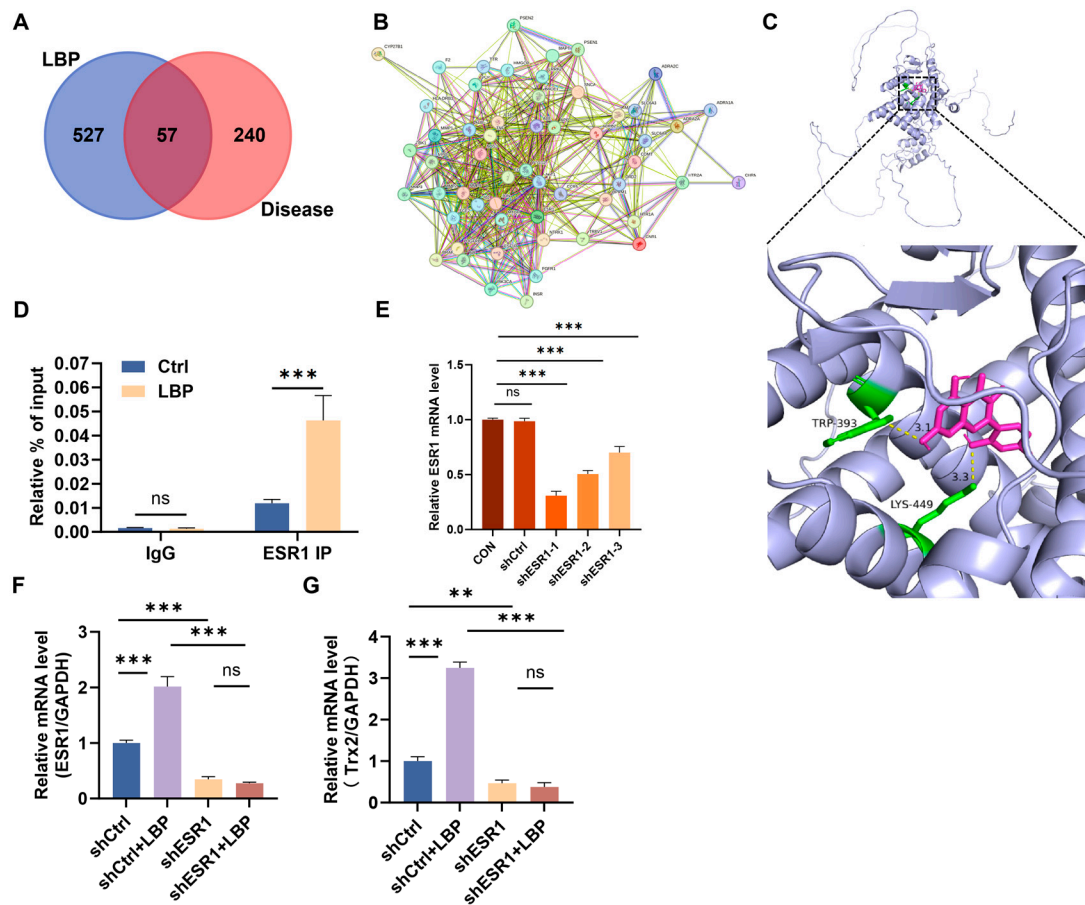


Figure 3: Target prediction of LBP in SNI and network pharmacological analysis of related target genes.

(A) Prediction of potential targets associated with LBP and SNI. (B) Protein-protein interaction network of 57 potential targets. (C) Molecular docking analysis of LBP and ESR1. (D) ChIP assay describes the impacts of ESR1 binding ability to Trx2 promoter compared with IgG control (n = 3 independent biological replicate experiments). (E) Quantification of ESR1 mRNA level in different groups of RSC96 cells after ESR1 knockdown (n = 3 independent biological replicate experiments). (F,G) Assessment of ESR1 and Trx2 mRNA levels in the shCtrl (RSC96 cells transfected with shCtrl), LBP (RSC96 cells treated with LBP and transfected with shCtrl), shESR1 (RSC96 cells transfected with shESR1), and shESR1 + LBP (RSC96 cells treated with LBP and transfected with shESR1) groups (n = 3 independent biological replicate experiments). ** $p < 0.01$, *** $p < 0.001$; ns, no significance. Abbreviations: LBP, *Lycium barbarum* polysaccharides; SNI, sciatic nerve injury; ChIP, chromatin immunoprecipitation; ESR1, estrogen receptor 1; Trx2, thioredoxin 2.

3.4 LBP Protects SCs against Oxidative Stress, Inflammation, and Apoptosis through Regulating ESR1

Next, we investigated whether ESR1 mediates the antioxidant and antiapoptotic effects of LBP in RSC96 cells. First, MMP analysis using JC-1 staining revealed an obvious trend that LBP maintained mitochondrial homeostasis in RSC96 cells, as indicated by preserved JC-1 aggregate formation, but this mitochondrial-protective effect was markedly diminished upon ESR1 knockdown (Fig. 4A). Notably, ATP detection also presented the same tendency (Fig. 4B). Furthermore, LBP suppressed the production of pro-inflammatory cytokines (TNF- α , IL-6, and IL-8) in RSC96 cells, and ESR1 knockdown reversed this anti-inflammatory action (Fig. 4C–E). Then, the proliferation abilities of RSC96 cells were evaluated, and the results substantiated that, although LBP sustained a degree of proliferative activity in RSC96 cells following ESR1 knockdown, the ablation of ESR1 expression significantly attenuated the promotional efficacy of LBP regarding Schwann cell proliferation (Fig. 4F,G). Western Blot analysis demonstrated that the inhibition of ESR1 resulted in elevated expression of Caspase-3 and BAX, alongside a reduction in Bcl-2 and Trx2 levels. These findings suggest that ESR1 mediates the critical antioxidative and antiapoptotic effects of LBP in SCs (Fig. 4H).

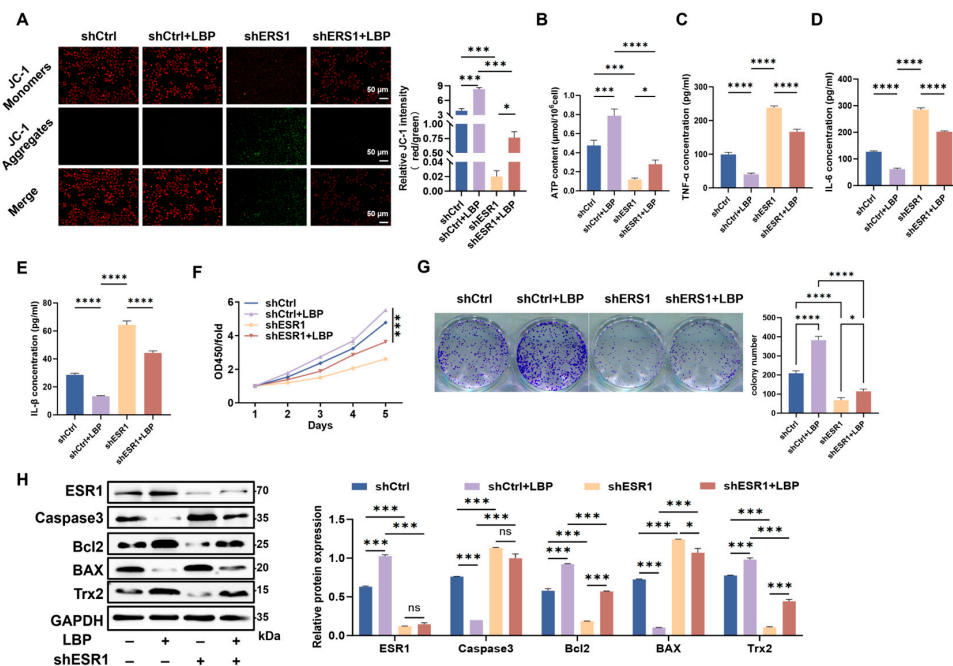


Figure 4: LBP promotes cell proliferation and inhibits cell apoptosis through ESR1. (A) Representative images of JC-1 staining in different groups and quantification of red/green fluorescence intensity (n = 3 independent biological replicate experiments). Scale bar = 50 μ m. (B) Cellular ATP content in the indicated groups (n = 3 independent biological replicate experiments). (C–E) Concentrations of pro-inflammatory cytokines TNF- α (C), IL-6 (D), and IL- β (E) in the culture supernatant (n = 3 independent biological replicate experiments). (F,G) Evaluation of proliferation ability of RSC96 cells by CCK-8 (F) and colony formation assays (G) (n = 3 independent biological replicate experiments). (H) Representative Western Blot images of cell apoptosis-related protein caspase3, Bcl2, BAX, and Trx2 protein changes and quantification in different groups (n = 3 independent biological replicate experiments). * p < 0.05, *** p < 0.001, **** p < 0.0001; ns, no significance. Abbreviations: LBP, *Lycium barbarum* polysaccharides; JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; ESR1, estrogen receptor 1; ATP, adenosine triphosphate; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; CCK-8, cell counting kit-8; Trx2, thioredoxin 2.

Compared to the physiological environment, the recovery of Schwann cell injury is more important for SNI. Therefore, we evaluated whether ESR1 has the same role in H_2O_2 -induced RSC96 cell injury. JC-1 staining and quantification revealed that H_2O_2 treatment markedly reduced MMP, as indicated by decreased JC-1 intensity, while LBP significantly restored MMP, but this protective effect was abolished by ESR1 knockdown (Fig. 5A). Consistently, LBP suppressed H_2O_2 -induced production of pro-inflammatory cytokines, and ESR1 silencing reversed this anti-inflammatory effect (Fig. 5B–D). CCK-8 and colony formation assays demonstrated that LBP promoted the proliferation of H_2O_2 -injured RSC96 cells, whereas ESR1 knockdown evidently attenuated this pro-proliferative effect (Fig. 5E,F). Western blot analysis further showed that LBP downregulated the expression of pro-apoptotic proteins (Caspase-3 and BAX) and upregulated anti-apoptotic protein (Bcl-2 and Trx2) levels in H_2O_2 -treated cells (Fig. 5G,H). Moreover, ESR1 silencing abrogated these changes, leading to re-elevation of Caspase-3 and BAX, and suppression of Bcl-2 and Trx2 (Fig. 5G,H). Additionally, ESR1 knockdown reversed the LBP-mediated inhibition of AIF and upregulation of Trx2 (Fig. 5I,J), further confirming the role of ESR1 in LBP-induced mitochondrial and anti-apoptotic protection.

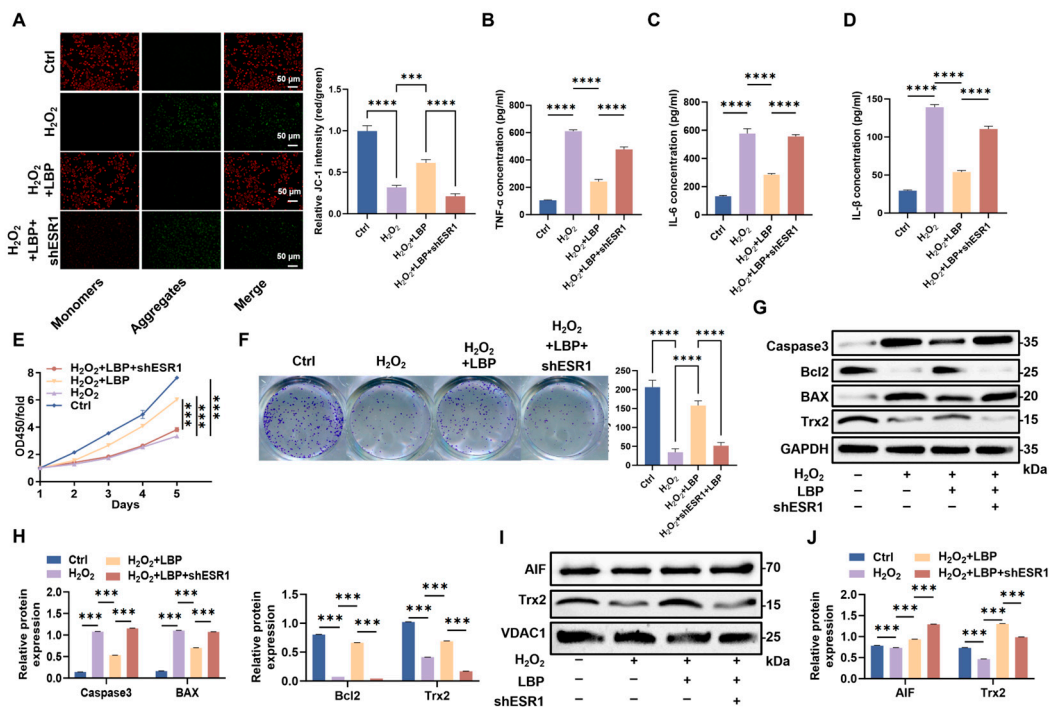


Figure 5: ESR1 mediates the antioxidant and anti-apoptotic effects of LBP in RSC96 cells. (A) Representative images of JC-1 staining in different groups and quantification of red/green fluorescence intensity ($n = 3$ independent biological replicate experiments). Scale bar = 50 μ m. (B–D) Concentrations of pro-inflammatory cytokines TNF- α (B), IL-6 (C), and IL- β (D) in the culture supernatant ($n = 3$ independent biological replicate experiments). (E,F) Evaluation of proliferation ability of RSC96 cells by CCK-8 (E) and colony formation (F) assays ($n = 3$ independent biological replicate experiments). (G,H) Representative Western Blot images of cell apoptosis-related protein caspase3, Bcl2, BAX, and Trx2 protein changes and quantification in different groups ($n = 3$ independent biological replicate experiments). (I,J) Representative Western Blot images of mitochondrial AIF and Trx2 protein changes and quantification in different groups ($n = 3$ independent biological replicate experiments). *** $p < 0.001$, **** $p < 0.0001$. Abbreviations: LBP, *Lycium barbarum* polysaccharides; JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; ESR1, estrogen receptor 1; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; CCK-8, cell counting kit-8; Trx2, thioredoxin 2.

3.5 LBP Improves SNI in Rats

Evaluation of the remedial efficacy of LBP concerning SNI was predicated on the induction of a rat SNI model, facilitating Sciatic Functional Index (SFI) quantification at the 4- and 8-week postoperative intervals. As delineated in Fig. 6A, a statistically significant reduction in SFI was evident in the Model group vis-à-vis the Sham control, thereby indicative of profound motor function compromise. In contrast, treatment with LBP was observed to improve the SFI score to a relatively high degree, which seems to suggest that a substantial recovery of function may have occurred. The H&E staining of the gastrocnemius muscle tissues appeared to show the presence of disordered nerve fiber arrangement, axon degeneration, and inflammatory cell infiltration in the Model group. These specific features were found to be alleviated to a certain extent following the administration of LBP (Fig. 6B). In addition, the use of toluidine blue staining seemed to show evidence of cellular pathology and necrosis in the SNI model group. However, it was observed that treatment with LBP partially reduced these types of pathological injuries (Fig. 6C). When these observations are taken together, the results seem to indicate that LBP might effectively facilitate the recovery of both structure and function after the induction of SNI in rats.

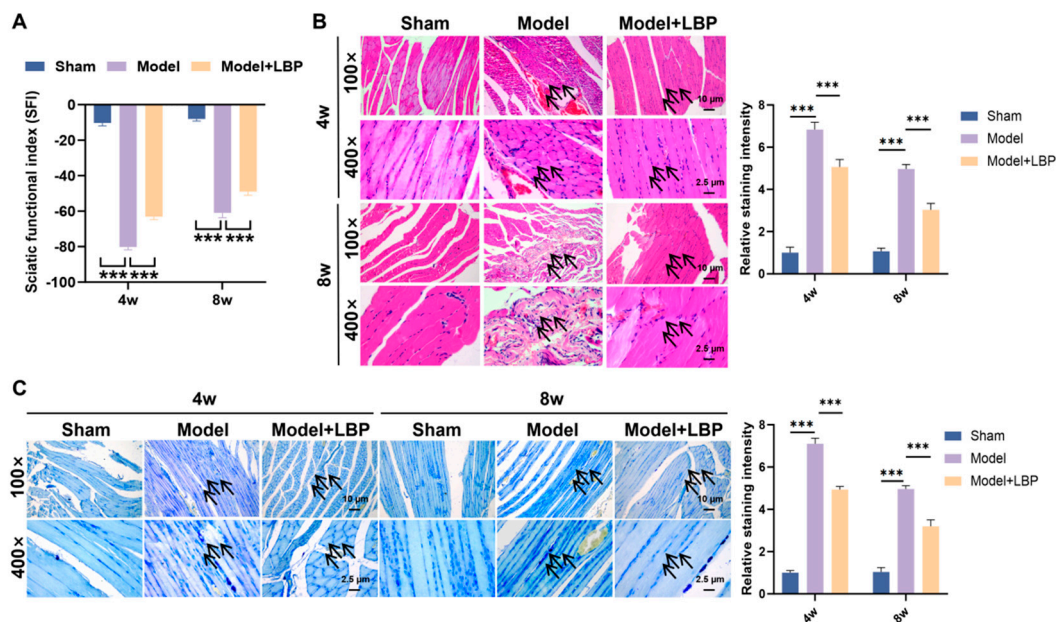


Figure 6: LBP improves SNI in rats. (A) SFI was measured to show motor function (n = 5 rats per group). (B) The results of H&E staining of the gastrocnemius muscle between groups (n = 5 rats per group). The black arrows in the Model group indicate disordered nerve fiber arrangement, swollen/degenerated axons, and inflammatory cell infiltration, while the black arrows in the Model + LBP group indicate relatively neat nerve fiber arrangement and reduced axon degeneration/inflammatory infiltration. (C) The results of toluidine blue staining of the sciatic nerve between groups (n = 5 rats per group). The black arrows in the Model group indicate discontinuous, fragmented, or lost myelin sheaths, while the black arrows in the Model + LBP group indicate more continuous and intact myelin sheaths. *** $p < 0.001$. Abbreviations: LBP, *Lycium barbarum* polysaccharides; SFI, sciatic functional index; H&E, hematoxylin and eosin.

4 Discussion

SNI is a prevalent peripheral nerve trauma that severely compromises patients' quality of life, with current therapeutic options such as surgery, glucocorticoids, and neurotrophic agents yielding unsatisfactory

outcomes [20,21]. SCs play a pivotal role in nerve repair and regeneration by initiating Wallerian degeneration, clearing debris, and remodeling the extracellular matrix, which are highly dependent on mitochondrial energy supply and redox homeostasis [22,23]. Mitochondrial dysfunction and excessive ROS accumulation post-SNI disrupt SC function, while concurrent inflammatory responses driven by pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) further exacerbate nerve damage, forming a pathological cascade that hinders regeneration [24–26]. Thus, targeting both mitochondrial stability and inflammation represents a promising therapeutic strategy for SNI.

LBP, a bioactive extract from *Lycium barbarum*, has been widely reported to exert antioxidant, anti-inflammatory, and neuroprotective effects in various neurological disorders, such as spinal cord injury and corticosterone-induced cognitive impairment [15,16,27]. However, its specific role and molecular mechanism in SNI remain unclear. In the present study, we established an *in vitro* SNI model using H₂O₂-induced RSC96 cells, which recapitulates the core pathological features of SNI, including mitochondrial dysfunction (reduced MMP and ATP production), oxidative stress (elevated ROS), excessive inflammation (increased pro-inflammatory cytokine secretion), and abnormal apoptosis. Our results demonstrated that LBP intervention significantly alleviated these pathological changes, restoring mitochondrial function, reducing ROS levels, suppressing pro-inflammatory cytokine release, and regulating the expression of apoptosis-related proteins (Caspase-3, BAX, Bcl-2). Notably, LBP also upregulated the mitochondrial antioxidant thioredoxin 2 (Trx2) and inhibited the release of apoptosis-inducing factor (AIF), highlighting its protective effects on mitochondrial redox homeostasis and anti-apoptotic capacity in SCs. *In vivo* validation using a rat SNI model further confirmed that LBP improved motor function (elevated SFI scores) and alleviated histological damage (reduced vascular congestion, vacuolization, and necrosis in sciatic nerve tissues), verifying its therapeutic potential for SNI.

To elucidate the molecular mechanism underlying LBP's protective effects, we employed network pharmacology and molecular docking, identifying estrogen receptor 1 (ESR1) as a key target of LBP with stable binding affinity. ChIP assays confirmed that LBP enhances the binding of ESR1 to the Trx2 promoter, promoting Trx2 transcription. Subsequent ESR1 knockdown experiments demonstrated that the beneficial effects of LBP, including improving mitochondrial function, inhibiting inflammation and apoptosis, and promoting SC proliferation, were significantly abrogated by ESR1 silencing. These findings indicate that LBP exerts its protective effects on SCs and SNI repair through the ESR1/Trx2 axis. Trx2, as a critical mitochondrial antioxidant, maintains redox balance by scavenging ROS and inhibiting mitochondrial permeability transition pore opening, while also suppressing AIF release to block caspase-independent apoptosis [28,29]. ESR1, a transcription factor involved in various neurological processes [30,31], mediates LBP-induced Trx2 upregulation, thereby integrating antioxidant, anti-inflammatory, and anti-apoptotic pathways to restore SC function.

It is worth noting that LBP may exert additional therapeutic effects through other pathways beyond the ESR1/Trx2 axis. Previous studies have shown that LBP can regulate the TLR4/NF- κ B and SIRT1/Nrf2 pathways to inhibit inflammation and enhance antioxidant capacity [32,33], which may synergize with the ESR1/Trx2 axis to amplify protective effects. For instance, Trx2 has been reported to inhibit NF- κ B activation by reducing ROS-induced I κ B α phosphorylation [34], suggesting potential crosstalk between the ESR1/Trx2 axis and inflammatory signaling pathways. However, the current study did not explore these interactions in depth, which constitutes a limitation. Additionally, direct comparisons between LBP and other neuroprotective agents (e.g., syringic acid, quercetin) are lacking, and the optimal dose, administration route, and long-term safety of LBP for clinical application require further investigation.

In conclusion, the present study demonstrates that LBP alleviates SNI by regulating the ESR1/Trx2 axis to restore SC function, including improving mitochondrial homeostasis, suppressing oxidative stress, inflammation, and apoptosis. These findings not only reveal the specific molecular mechanism of LBP in SNI treatment but also provide a novel strategy for the development of therapeutic agents targeting SC mitochondrial function and inflammation. Future studies should focus on exploring the crosstalk between the ESR1/Trx2 axis and other signaling pathways, optimizing LBP's clinical application parameters, and conducting large-scale clinical trials to validate its efficacy.

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Availability of Data and Materials: The data that support the findings of this study are available from the Corresponding Author, [Bowen Zhang], upon reasonable request.

Ethics Approval: This study was approved by the Institutional Animal Care and Use Committee of People's Hospital of Ningxia Hui Autonomous Region (Approval No. [2023]-NZR-103). And we confirmed this study is reported in accordance with ARRIVE guidelines.

Conflicts of Interest: The authors declare no conflicts of interest.

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