Resveratrol-enhanced SIRT1-mediated osteogenesis in porous endplates attenuates low back pain and anxiety behaviors

Xiao Lv | Songfeng Chen | Feng Gao | Binwu Hu | Yongkui Wang | Shuangfei Ni | Hongwei Kou | Zongmian Song | Xiangcheng Qing | Shangyu Wang | Hongjian Liu | Zengwu Shao

1Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
2Department of Orthopaedics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Correspondence
Hongjian Liu, Department of Orthopaedics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China. Email: fccliuhj@zzu.edu.cn
Zengwu Shao, Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China. Email: szwpro@163.com

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Abstract
Low back pain (LBP) is a major clinical problem that lacks effective treatments. The sensory innervation in porous vertebral endplates and anxiety contributes to spinal hyperalgesia. We hypothesized that SIRT1 activator resveratrol alleviates LBP and anxiety via promotion of osteogenesis in the porous endplates. The hyperalgesia and anxiety-related behaviors; sensory innervation, inflammation and porosity of endplates; and osteogenic/osteoclastic factors expression were measured following resveratrol treatment after lumbar spine instability (LSI) surgery. To explore whether resveratrol promotes endplates osteogenesis and thus alleviates LBP through activation of SIRT1 in the osteoprogenitor cells of endplates, SIRT1−/− mice were employed. Additionally, the levels of inflammation markers, phosphorylation of cAMP response element-binding protein (pCREB), and brain-derived neurotrophic factor (BDNF) in hippocampus were evaluated. After 4 or 8 weeks LSI surgery, the mice suffered from hyperalgesia and anxiety, which were efficiently attenuated by resveratrol at 8 weeks. Resveratrol treatment-enhanced osteogenesis and decreased endplates porosities accompanied with the reduction of TNFα, IL-1β, and COX2 levels and CGRP+ nerve fibers innervation in porous endplates. Resveratrol-mediated endplates osteogenesis, decreased endplates porosities, and analgesic and antianxiety effects were abrogated in SIRT1−/− mice. Furthermore, resveratrol relieved inflammation and increased pCREB and BDNF expression in the hippocampus after 8 weeks, which alleviate anxiety-related behaviors. This study provides that resveratrol-mediated porous endplates osteogenesis via the activation of SIRT1 markedly blocked sensory innervation and inflammation in endplates, therefore, alleviating LSI surgery-induced LBP and hippocampus-related anxiety.

KEYWORDS
anxiety, low back pain, osteogenesis, porous endplates, resveratrol, SIRT1

Abbreviations: BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; IL-1β, interleukin-1β; LBP, low back pain; pCREB, phosphorylation of cAMP response element-binding protein; SIRT1, Sir2type 1; TNFα, tumor necrosis factor-α.

Xiao Lv and Songfeng Chen have contributed equally to this work.

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1 | INTRODUCTION

Low back pain (LBP) affects up to 80% of people at some point during their lifetime and is the leading cause of disability worldwide.1,2 Traditionally, LBP mainly occurs in the elderly, but it has gradually shown a trend of affecting younger individuals in recent years.3,4 The current therapeutic options for LBP are oriented either toward conservative treatments or surgical procedures, but none of these approaches solve the problem fundamentally.5 To develop effective therapeutic targets, it is imperative to identify the cause and explore the underlying mechanism of LBP.

Intervertebral disk (IVD) degeneration-related disease is strongly correlated with LBP.6,7 The vertebral endplates serve as the interface of the IVD and vertebral bodies and facilitate nutrient transport between these structures. Growing evidence indicates that endplates lesions are more prevalent in cohorts of patients with LBP than in healthy individuals.8-10 During the process of endplates degeneration, the numbers of osteoclasts in the endplates are apparently elevated, which results in increased porosities and accumulation of inflammatory factors in the endplates.11-13 Porous endplates are more extensively innervated by sensory nerve fibers than the normal endplates.14 Progressively increased porosities and decreased osteogenesis in endplates substantially exacerbate LBP.11,12 Therefore, the enhancement of osteogenesis within porous endplates might prevent the local immune inflammatory response and block sensory innervation in endplates, and could be expected to provide a brand-new strategy for the treatment of LBP.

Sir2uin type 1 (SIRT1), a class III histone deacetylase, is closely involved in the regulation of osteogenic differentiation.15,16 For instance, mechanical stretch induces antioxidative responses and osteogenic differentiation in human mesenchymal stem cells (MSCs) through activation of the AMP-activated protein kinase-SIRT1 signaling pathway.17 Sun et al reported that the overexpression of SIRT1 in MSCs-enhanced osteoblastic bone formation and protected against bone loss, thus ameliorating skeletal defects.18 Resveratrol, a SIRT1 activator, is a natural polyphenol that is present in many plant-based foods, most notably red wine.19 A recent study documented that resveratrol treatment resulted in decreased adipogenesis and increased osteogenesis in the separation-based anorexia model.20 Furthermore, resveratrol noticeably alleviated the disk degeneration-mediated LBP;21 however, only simple spinal pain-related behaviors were evaluated, and the underlying mechanism remains elusive. Hence, it is logical to speculate that resveratrol exerts its analgesic effect by promoting osteogenesis in porous endplates.

Clinical studies have reported that patients with LBP often exhibit obsessive-compulsive disorder, panic disorder, and anxiety.22,23 Anxiety disorders aggravate the occurrence and development of LBP, and the relationship between LBP and anxiety has recently attracted growing attention.24,25 Resveratrol apparently alleviated multiple stress-induced anxiety-related behaviors.26,27 Could the antianxiety effect of resveratrol contribute to LBP relief? The hippocampus, which is sensitive to stress, has recently received increasing attention in anxiety.28,29 Resveratrol promoted the phosphorylated of cAMP response element-binding protein (pCREB) and brain-derived neurotrophic factor (BDNF) synthesis in hippocampus, which are positively correlated with the relief of anxiety.30,31 In addition, inflammatory infiltration in the hippocampus is viewed as central to the development of anxiety.32,33 Given that hippocampal inflammation mediates or exacerbates anxiety, we hypothesized that hippocampal inflammation may exacerbate the experience of LBP after LSI surgery in mice.

In the current study, we aimed to clarify the underlying mechanism of the resveratrol-induced attenuation of LBP and anxiety after LSI surgery. Resveratrol treatment strongly relieved LSI surgery-induced LBP and anxiety; importantly, resveratrol dramatically increased osteogenesis, therefore, blocking sensory innervation and inflammatory infiltration within porous endplates, and these effects were abrogated in the SIRT1OSX−/− mice. Additionally, resveratrol-enhanced pCREB activation BDNF synthesis and attenuated hippocampal inflammation. We conclude that resveratrol-enhanced SIRT1-mediated osteogenesis in porous endplates attenuates LBP and anxiety.

2 | MATERIALS AND METHODS

2.1 | Mice model and in vivo treatments

All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Animal Protection Guidelines of Tongji Medical College of Huazhong University of Science and Technology, China. C57BL/6J male mice were maintained under specific pathogen-free (SPF) conditions with a 12 light/12 dark cycle and free access to food and water, room temperature, and relative humidity were maintained at 22 ± 1°C and 50 ± 20%, respectively, no more five mice were maintained per cage. Osx-Cre mice (006361) and SIRT1flox/flox mice (NM-CKO-190037) were purchased from Shanghai Model Organisms (Shanghai, China). Heterozygous Osx-Cre were crossed with a SIRT1floxed mouse, the offspring were intercrossed to generate followed genotype: WT, Osx-Cre, SIRT1floxed, and Osx-Cre: SIRT1floxed. Specific knockout SIRT1 in the osteoprogenitor cells Osx-Cre: SIRT1floxed are referred to SIRT1OSX−/− in the manuscript, and the littermate control SIRT1floxed referred to SIRT1wt in the manuscript. The mice were genotyped by PCR analyses of genomic DNA extracted from tail samples from 1-month-old
mice. The genotyping primers were as follows: Osx-Cre:
forward: GAGAATAGGAACTTCGGAATAGTAAC,
reverse: CCCTGGAAGTGACTAGCATTG. SIRT1\text{flox}/\text{flox}:
forward: AGGAATCCCACAGGAGACAG, reverse:
GGTTAAGATTAGCCCATTAAAGC.

Two-month-old mice were anesthetized and underwent
LSI surgery by the resection of the lumbar 3\textsuperscript{rd}-5\textsuperscript{th} spinous
processes as well as the interspinous and supraspinous lig-
ments to create instability of the lumbar spine according to
the previous studies.\textsuperscript{12} We only performed detachment of the
posterior paravertebral muscles at level L3-5 vertebrae.

The mice were administered either 40 mg/kg body weight
resveratrol (R5010, Sigma Millipore, St. Louis, MO, USA)
or an equivalent volume of DMSO (vehicle group) by intra-
peritoneal injection once per day, starting on the day after
the LSI surgery was performed. The mice were euthanized
at 2, 4, and 8 weeks post-LSI surgery. Spine, DRG, and brain
samples were collected.

2.2 | Behavioral tests

All the behavioral tests were performed once before the
surgery and weekly after the surgery. All the tests were per-
formed by the same investigator blinded to the experimen-
tal conditions. The test mice were exposed to the behavioral
test equipment for at least 1 hour per day for 3 days prior
to the formal tests. All tests were performed between 10:00
and 16:00 during the light cycle. All equipment was cleaned with
75% ethanol following each test.

2.2.1 | Running wheel activity

RW activity utilized the well-established fact that mice have
a spontaneous tendency to run. The RW activity equipment
(Campden Instruments, Loughborough, UK) included a
wheel with a diameter of 23 cm and a lane width of 5 cm in
the mouse cages, and software was used to record the spon-
taneous activity of the wheel. The test mice were given free
access to water and food in their cages. The active time, dis-
tance traveled, mean speed, and max speed within a 48 h pe-
riod were recorded for each mouse.

2.2.2 | Vocalization threshold test

Pressure hyperalgesia was measured by vocalization. We ap-
plied the force gauge (SMALGO algometer; Bioseb, Pinellas
Park, FL, USA) was used to record the curve of pressure
force with a maximum pressure of 500 g. The mean value
was calculated as the nociceptive threshold.

2.2.3 | Open-field test

The open-field test is a classic assessment to measure anxiety-
related behavior that provides the opportunity to systemati-
cally assess novel environmental exploration. A large square
room (34 × 34 cm) with transparent plexiglas walls were
used. The individual test mice were placed in the center of the
room after 1 hour of adaptation. The movement of mice in the
room, the total distance traveled, and the time spent in the
center area (15 × 15 cm) over a 10 minutes period were
recorded.

2.2.4 | Elevated plus maze test

The elevated plus maze test was performed. Briefly, the maze
(O’Hara&Co., Tokyo, Japan) consisted of two open arms
(25 × 25 cm) and two closed arms with a common central
platform (5 × 5 cm) that is placed at a height of 1 m above
the floor. The individual test mice were placed on the center
platform, and the time spent in the open arms over a 10 min-
utes period was recorded.

2.3 | Histology, immunofluorescence, and
histomorphometry

The lumbar spine, DRG, and brain samples were collected
and fixed overnight in 4% paraformaldehyde, and the lum-
bar spine samples were decalcified with 10% EDTA (pH
7.4-7.6) (EDS, Sigma Millipore, St. Louis, MO, USA) for
21 days with constant shaking at 4°C. The solution was
changed every day. The samples were then dehydrated with
30% sucrose for 24 hours and embedded in optimal cutting
temperature (OCT) compound (Sakura Finetek, Torrance,
CA, USA) or paraffin. Four-micrometer-thick coronal-
oriented sections of the L4-5 lumbar spine were prepared
for Safranin-O/Fast Green staining, and tartrate-resistant
acid phosphatase (Trap) staining was performed according
to the kit protocol (Sigma Millipore, St. Louis, MO, USA).
Immunostaining was performed using a standard protocol.
Briefly, 20- and 40-μm-thick coronal-oriented sections of
the lumbar spine were prepared for cell and nerve staining,
respectively. The sections were blocked with bovine serum
albumin (BSA, Sigma Millipore, St. Louis, MO, USA) with
0.1% Triton-100 (Sigma Millipore, St. Louis, MO, USA) for
1 hour at room temperature and incubated overnight at 4°C
with the following primary antibodies: anti-COX2 (ab15191,
Abcam, Cambridge, MA, USA), anti-osterix (ab22552, Abcam, Cambridge, MA, USA), anti-CGRP (ab81887, Abcam, Cambridge, MA, USA), anti-pCREB (ab32096, Abcam, Cambridge, MA, USA), and anti-BDNF (ab108319, Abcam, Cambridge, MA, USA). Fluorescence-conjugated secondary antibodies were used to detect the fluorescent signals after counterstaining with DAPI (H-1500, Vectorlab, Burlingame, CA, USA). A Zeiss LSM780 confocal microscope or Olympus microscope was used for image capture. The quantitative histomorphometric analysis was executed by the OsteoMeasure Software (OsteoMetric, CA, USA) in a blind condition.

Double-labeling assay was employed to measure the dynamic bone formation. 0.1% calcein (C0875, Sigma-Aldrich, St. Louis, MO, USA) in PBS was injected at the concentration of 10 mg per kg subcutaneously 7 days and 1 day before sacrifice. Undecalified spine slices were captured with fluorescence microscope. Endplates bone formation were measured in three randomly selected visual fields.

2.4 | μCT

The lumbar vertebrae were harvested and fixed in 4% paraformaldehyde overnight, rinsed with PBS solution, and prepared for high-resolution μCT (Micro-CT, Skyscan1174, USA). The voltage of the scanning procedure was 55 kV, and 181 μA was used. The resolution was set to 9 μm per pixel to detect endplates and vertebrae. The reconstruction software NRecon and SkyScan and the analysis software CTAn and SkyScan were used. Three-dimensional histomorphometric coronal sections of caudal L4-5 endplates were analyzed. The three-dimensional parameters Tb.Sp and porosity percentage were analyzed for the L5 caudal endplates. Five consecutive coronal sections from the endplates were used for the three-dimensional model visualization software (CTVol v2.0 (SkyScan)).

2.5 | Quantitative real-time polymerase reaction chain (RT-PCR)

The total RNA was purified from the endplates using TRIzol (15596026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RT-PCR was performed using SYBR Green Power PCR Master Mix (A25777, Invitrogen, Carlsbad, CA, USA) on a CFX Connect instrument (Bio-Rad, Hercules, CA, USA). GAPDH amplification was used as an internal control. The relative expression level of each gene was calculated by the 2^−ΔΔCT method.

2.6 | ELISA assays

The concentrations of PGE2 and IL-1β in the L4-5 endplates and the concentration of BDNF in the hippocampus were measured by a PGE2 ELISA kit (514010, Cayman Chemical, Ann Arbor, MI, USA), IL-1β ELISA kit (BMS6002, Thermo Fisher, Waltham, MA, USA), and BDNF ELISA kit (dy248, R&D System, Minneapolis, MN), respectively. The concentration of Netrin-1 in the L4-5 endplates was also determined by a Netrin-1 ELISA kit (EKC37454, Biomatik, Wilmington, DE, USA).

2.7 | Statistics

Data are presented as the means ± the standard errors of the mean (SEM). We used two-tailed Student’s t tests for comparisons between two groups and one-way ANOVA for comparisons among multiple groups. The significance level was set at P < .05. No statistical method was used to predetermine the sample size. The investigators were blinded to the allocation groups during the experiments and the assessment of the results. All data analyses were performed by SPSS v22.0 (IBM Corp, Armonk, NY).

3 | RESULTS

3.1 | Resveratrol attenuates LSI surgery-induced hyperalgesia and anxiety behaviors

To examine the effects of resveratrol in relieving the symptoms of LBP and anxiety, we administrated resveratrol to the mice for 2, 4, and 8 weeks followed by LSI surgery. The mice running wheel activity was used to measure hyperalgesia-related behaviors. The active time, distance traveled, mean speed, and maximum speed were almost equivalent among mice from the sham, vehicle, and resveratrol groups at 2 weeks post-LSI surgery. Each parameter decreased in the
vehicle group at 4 and 8 weeks post-LSI surgery. Resveratrol treatment rescued the decreased trend at 8 weeks, whereas no significant improvement was observed at 4 weeks post-LSI surgery (Figure 1A-D). We also applied low back force directly to the L4-5 disk region. Gradually decreased vocalization thresholds were recorded in the vehicle group; interestingly, mice treated with resveratrol exhibited markedly improved pressure tolerance at both 4 and 8 weeks post-LSI surgery (Figure 1E).

Clinical evidence has revealed that anxiety exacerbates LBP.24,25 We first introduced the open-field and elevated plus maze tests to evaluate anxiety-related behaviors in mice after LSI surgery. Intriguingly, we found that the anxiety behaviors of the vehicle group were elevated only at 4 and 8 weeks post-LSI surgery (Figure 1F-I). The resveratrol treatment dramatically alleviated the anxiety behaviors at 8 weeks post-LSI surgery, as evidenced by the greater distance and time traveled in the center area as well as the greater amount of time spent in the open arms (Figure 1F-I). These results indicated that resveratrol effectively relieved LBP and anxiety at 8 weeks post-LSI surgery.

### 3.2 Resveratrol attenuates inflammation and sensory innervation in endplates

To investigate the analgesic mechanism of resveratrol, we measured the expression of inflammatory factors and the innervation of nociceptive C nerve fibers in the endplates. The expression of PGE2 in the endplates was elevated at 4 and 8 weeks post-LSI surgery. The resveratrol-treated group showed a dramatic decrease in PGE2 levels at 8 weeks but did not present the statistical difference at 4 weeks compared with the vehicle group (Figure 2A). The levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) in the endplates were also measured. Similarly, resveratrol treatment strongly downregulated the expression of IL-1β and TNFα in the endplates at 8 weeks post-LSI surgery, while no significant decrease was observed in the 4 weeks group (Figure 2B,C). Therefore, we chose 8 weeks LSI surgery in the following experiments.

The expression of cyclooxygenase 2 (COX2) in the endplates dramatically decreased in the resveratrol treatment group compared with that in the vehicle group at 8 weeks post-LSI surgery, as evidenced by the immunostaining of COX2 in the endplates (Figure 2D,E). Moreover, immunostaining revealed the abundant presence of calcitonin gene-related peptide (CGRP), a marker of nociceptive C nerve fibers innervated into porous endplates at 8 weeks post-LSI surgery, which were largely eliminated by resveratrol (Figure 2F,G). A significant reduction of the CGRP+ dorsal root ganglion (DRG) neurons was observed by immunostaining in the resveratrol treatment group at 8 weeks post-LSI surgery (Figure 2H,I). Together, these results suggested that resveratrol attenuated inflammation and sensory innervation in endplates at 8 weeks post-LSI surgery.

### 3.3 Resveratrol attenuates LSI surgery-induced endplates porosities

To intuitively observe the morphological changes of endplates after LSI surgery, microcomputed tomography (μCT) and Safranin-O/Fast Green (SOFG) staining were performed (red, cartilage; green, bone). We discovered increasing size of porosities within the L4-5 caudal endplates relative to sham group, as evidenced by three-dimensional μCT (Figure 3A). A significant reduction of porosities was detected after resveratrol treatment as determined by μCT, trabecular bone separation distribution (Tb.Sp), and porosities percentage (Figure 3A-C).

Moreover, the SOFG staining showed that green-stained bone matrix surrounded the marrow-like hypertrophy porosities within the endplates at 8 weeks post-LSI surgery. We observed marked reduction of porosities in the endplates, which filled with green-stained bone matrix after resveratrol treatment, these phenomena indicated that resveratrol effectively promoted bone formation within the porous endplates (Figure 3D). We assessed the endplates score based on the pathological changes of endplates such as bony sclerosis, structural disorganization, and neovascularization. Resveratrol treatment significantly reduced the elevated endplates score induced by LSI surgery (Figure 3E). Besides, the RT-PCR was utilized to evaluate the expression of anabolic factors Aggrecan and Collagen II as well as the degeneration markers MMP3 and MMP13 in the endplates. Resveratrol treatment significantly attenuated the LSI surgery-induced reduction in Aggrecan and Collagen II expression (Figure 3F,G), whereas obviously downregulated MMP3 and MMP13 levels relative to vehicle group (Figure 3H,I). These results suggested that resveratrol accelerates endplates anabolic metabolism, and attenuates endplates porosities after LSI surgery.

### 3.4 Resveratrol promotes osteogenesis in the porous endplates after LSI surgery

To evaluate whether resveratrol promoted osteoblastic bone formation in the porous endplates, we performed immunofluorescent staining of osterix, a marker of osteoprogenitor cells. Resveratrol treatment significantly boosted the expression of osterix+ osteoprogenitor cells in the porous endplates (Figure 4A,B). The bone formation and mineral apposition rates were also appreciably increased in the resveratrol group, as evidenced by the calcein double-labeling
FIGURE 1  Resveratrol attenuates LSI surgery-induced hyperalgesia and anxiety behaviors. A-D, The quantitative analysis of running wheel activity, including active time (A), distance traveled (B), mean speed (C), and max speed (D) for 48 h determined by the percentage of sham surgery mice at the corresponding time points 40 mg/kg/d resveratrol and vehicle treatment after 2, 4, and 8 weeks LSI surgery. E, The quantitative analysis of the vocalization threshold test for lumbar spine pressure hyperalgesia at the corresponding time points in resveratrol- and vehicle-treated mice after LSI surgery. F-I, Quantitative analysis of anxiety-related behavior test, in the open-field test F-G, including the time spent in the center of the open-field and the distance traveled in the field at the corresponding time points. The elevated plus maze test (H-I) measured the time spent on the open arms by the resveratrol- and vehicle-treated mice after the LSI surgery at the corresponding time points. *P < .05, **P < .01. (Student’s t test)
FIGURE 2  Resveratrol attenuates inflammation and sensory innervation in endplates. A-C, ELISA analysis of the PGE2, IL-1β, and RT-PCR analysis of expression of TNFα in the lumbar endplates lysates from mice in the sham, vehicle and 40 mg/kg/d resveratrol treated groups at 4 and 8 weeks post-LSI surgery. D-E, Representative images of the immunofluorescence and quantitative analysis of COX2+ cells (green) in endplates from mice in the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar: 100 μm. F-G, Representative images of the immunofluorescence and quantitative analysis of CGRP+ sensory fibers (green) in the endplates from mice in the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar: 50 μm. H-I, Representative images of the immunofluorescence and quantitative analysis of the CGRP+ sensory neurons (green) in L2-4 DRGs from mice in the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar: 100 μm. N ≥ 6 per group. *P < .05, **P < .01. (Student’s t test)
assay (Figure 4C,D). Accordingly, the osteoclast number in the endplates was significantly decreased in the resveratrol group (Figure 4E,F). Slit-3, which is mainly derived from osteoblasts to block the nerve innervation, displayed intensely increased in the endplates after resveratrol treatment (Figure 4G). Consistently, resveratrol treatment strongly upregulated

![Figure 3](image)
FIGURE 4  Resveratrol promotes osteogenesis in the porous endplates after LSI surgery. A-B, Representative immunofluorescence images of the Osterix staining in the L4-5 endplates and the quantitative analysis of the number of osterix+ cells per section from the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar, 20 μm. C-D, Representative images of the calcein double-labeling of porous endplates with quantification of the mineral apposition rate from the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery, distance between white arrowheads were measured. E-F, Representative images of Trap staining of the endplates and quantitative analysis of the osteoclast number per section from the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar, 50 μm. G-H, The RT-PCR analysis of Slit3 and Runx2 expression in the L4-5 endplates from the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. I, The quantitative ELISA evaluation of Netrin-1 concentration in the L4-5 endplates from the sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. N ≥ 6 per group. *P < .05, **P < .01. (Student’s t test)
osteoblastic factor Runx2 expression compared with the vehicle group (Figure 4H). The expression of Netrin-1, which is secreted by osteoclasts to guide sensory innervation in the endplates, was also measured. After resveratrol treatment, Netrin-1 expression and the number of osteoclasts decreased in the endplates at 8 weeks post-LSI surgery (Figure 4I). These results demonstrated that resveratrol potently promotes osteogenesis within the porous endplates and blocks the sensory innervation in the endplates.

3.5 | Resveratrol-mediated analgesic and antianxiety effects via activation of SIRT1 in the endplates osteoprogenitor cells

To explore the mechanism of resveratrol in relieving LBP and anxiety, SIRT1OSX−/− mice, specifically knockout SIRT1 in the osterix positive osteoprogenitor cells of endplates were employed. We crossed OSX-Cre with SIRT1 floxed (SIRT1wt) mice to generate the SIRT1OSX−/− mice. SIRT1 expression in the endplates was examined to evaluate the knock out efficiency. Indeed, SIRT1 expression was significantly decreased in the SIRT1OSX−/− compared with SIRT1wt mice (Figure 5A). Immunostaining showed that CGRP+ sensory nerves in the endplates were severely depleted in the resveratrol-treated SIRT1wt mice at 8 weeks post-LSI surgery. However, the resveratrol-treated SIRT1OSX−/− mice still exhibited abundant CGRP+ sensory nerves in the endplates (Figure 5B). The results displayed that SIRT1OSX−/− mice abrogated the effect of resveratrol played in blocking sensory nerves innervation in the endplates. We further performed behavioral tests related to hyperalgesia and anxiety. In the SIRT1OSX−/− mice, resveratrol treatment failed to alleviate the LSI surgery-induced LBP, as evidenced by the notably decreased active time, mean speed, max speed, and distance traveled relative to sham group and resveratrol-treated SIRT1wt mice (Figure 5C-F). Accordingly, the open-field and elevated plus maze tests revealed that SIRT1OSX−/− mice abrogated resveratrol-mediated antianxiety effect at 8 weeks post-LSI surgery (Figure 5G). Thus, resveratrol-mediated activation of SIRT1 in endplates osteoprogenitor cells contributed to the analgesic and antianxiety effects after LSI surgery.

3.6 | Resveratrol promotes osteogenesis in porous endplates through activation of SIRT1 in the osteoprogenitor cells

To assess whether resveratrol promotes osteogenesis within the porous endplates through the activation of SIRT1 in osteoprogenitor cells, we measured the porosity of L4-5 caudal endplates. After resveratrol treatment, the porosities of the endplates decreased significantly in the SIRT1wt mice at 8 weeks post-LSI surgery, whereas resveratrol failed to prevent endplates porosities in the SIRT1OSX−/− mice, as evidenced by increased porosities and Tb.Sp (Figure 6A-C). Moreover, the SOFG images showed that the resveratrol-treated SIRT1OSX−/− mice still developed large porosities and exhibited high endplates score after LSI surgery (Figure 6D,E). Then, we performed immunostaining of osterix in the L4-5 endplates. A dramatically increased expression of osterix was detected in the resveratrol-treated SIRT1wt mice at 8 weeks post-LSI surgery. However, osterix expression was hardly observed in the porous endplates in the SIRT1OSX−/− mice (Figure 6F,G). Consistently, compared with SIRT1wt, nerves repelled factor Slit-3 expression in the endplates significantly decreased after resveratrol treated in SIRT1OSX−/− mice (Figure 6H). Additionally, endplates Aggrecan expression in the resveratrol-treated SIRT1OSX−/− mice decreased at 8 weeks post-LSI surgery (Figure 6I), while the expression of catabolic factor MMP13 in the endplates significantlyincreased (Figure 6J). Collectively, these results demonstrated that resveratrol-mediated osteogenesis in the porous endplates through activation of SIRT1 in the endplates osteoprogenitor cells after LSI surgery.

3.7 | Resveratrol alleviates hippocampal inflammation and enhances pCREB activation, BDNF expression in the hippocampus

We examined the expression of TNFα, IL-1β, and IL-6 in the hippocampus by RT-PCR. The TNFα, IL-1β, and IL-6 levels in the hippocampus were appreciably elevated at 8 weeks post-LSI surgery (Figure 7A-C). As expected, resveratrol treatment attenuated TNFα, IL-1β, and IL-6 expression (Figure 7A-C). Decreased pCREB activation and BDNF expression in the hippocampus aggravates anxiety under certain circumstances. Immunostaining revealed that the signal intensity of pCREB visibly decreased at 8 weeks post-LSI surgery and was largely restored following resveratrol treatment (Figure 7D). Likewise, resveratrol treatment appreciably rescued the LSI surgery-induced reduction of BDNF expression at 8 weeks post-LSI surgery (Figure 7E,F). These results suggested that resveratrol efficiently alleviated hippocampal inflammation and restored pCREB activation and BDNF expression in the hippocampus after LSI surgery.

4 | DISCUSSION

Effective therapy for LBP is hindered by its multifactorial etiology and the multiple tissues involved. Nowadays, efforts to identify the causes of LBP have increasingly
focused on the sensory innervation in the degenerative vertebral endplates. In current study, we first reported that resveratrol markedly alleviated LSI surgery-induced LBP and anxiety, which might be attributed to SIRT1 activation-mediated osteogenesis and the resultant reduction of the sensory innervation in porous endplates.

**FIGURE 5** Resveratrol-mediated analgesic and antianxiety effects via activation of SIRT1 in the endplates osteoprogenitor cells. A, The RT-PCR analysis of SIRT1 expression in the L4,5 endplates from SIRT1OSX−/− and relative control group SIRT1wt mice treated with resveratrol for 1 month. B, Representative immunofluorescence images of CGRP+ sensory fibers (red) in the L4,5 endplates from the SIRT1OSX−/− and SIRT1wt mice treated with resveratrol for 1 month. C-F, The quantitative analysis of the running wheel activities, including the active time (C), mean speed (D), max speed (E), and distance traveled (F), of the resveratrol-treated SIRT1OSX−/− and SIRT1wt mice over a 48 hours period at 8 weeks post-LSI surgery shown as percentages of the values obtained from the sham littermate controls. G-H, The quantitative analysis of open-fields test, including the time spent (G) in the center of the open-field and the distance traveled (H) in the field by the resveratrol-treated SIRT1OSX−/− and SIRT1wt mice after 8 weeks LSI surgery. I-J, The elevated plus maze test measured the time spent on the open arms by the resveratrol-treated SIRT1OSX−/− and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. N ≥ 10 per group. *P < .05, **P < .01. (Student’s t test for A, two-way ANOVA for C-J)
The endplates are vulnerable to suffered from lesions, and termed as the “weak link” of the lumbar spine. Endplates lesions cause considerable reduction in the disk’s proteoglycan and lead to elevated catabolic enzymes and pro-inflammatory cytokines, resulting in degenerative changes. It is believed that endplates lesions leads to a persistent inflammatory state in and nearby the disk, therefore, becoming an important pain generating source. In the present study, through μCT and SOFG staining, increasing porosities within the L₄₅ caudal endplates were observed at 8 weeks post-LSI surgery. A noticeable reduction in endplates porosities was detected following resveratrol treatment. Additionally, we discovered...
FIGURE 6  Resveratrol promotes osteogenesis in porous endplates through activation of SIRT1 in the osteoprogenitor cells. A, Representative coronal images of three-dimensional high-resolution μCT of mouse L₄-₅ caudal endplates from the resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. Scale bar, 1 mm. B-C, The quantitative analysis of trabecular bone separation (Tb.Sp) and the total porosity of caudal endplates from the resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. D, Representative images of the Safranin-O/Fast Green staining of coronal sections of L₄-₅ caudal endplates from resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. Scale bar, 50 μm. E, Endplate score analysis according to the Safranin-O/Fast Green staining in panel D from the resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. F-G, Representative immunofluorescence images of the Osterix staining in the L₄-₅ endplates and the quantitative analysis of the number of osterix+ cells per section from the resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. Scale bar, 20 μm. H-J, The RT-PCR analysis of Slit3, Aggrecan, and MMP13 expression in the L₄-₅ endplates from the resveratrol-treated SIRT1OSX⁻/⁻ and SIRT1wt mice and the sham littermate controls at 8 weeks post-LSI surgery. N ≥ 6 per group. *P < .05, **P < .01. (Two-way ANOVA)

FIGURE 7  Resveratrol alleviates hippocampal inflammation and enhances pCREB activation, BDNF expression in the hippocampus. A-C, The RT-PCR analysis of the expression of the inflammatory mediators TNFα (A), IL-1β (B), and IL-6 (C) in the hippocampus of resveratrol-treated and control mice at 8 weeks post-LSI surgery. D, Representative images of the pCREB immunostaining at the dentate gyrus of the hippocampus from sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar, 200 μm. E-F, Representative images of the BDNF immunostaining at the dentate gyrus of the hippocampus and the quantitative ELISA analysis of BDNF in the hippocampus from sham, vehicle, and resveratrol groups at 8 weeks post-LSI surgery. Scale bar, 200 μm. *P < .05. (Student’s t test)
that resveratrol appreciably boosts the expression of osterix+ osteoprogenitor cells in porous endplates. The osteoclasts in the endplates were considerably decreased in the resveratrol group. These results confirmed that resveratrol improved osteogenesis in the porous endplates after LSI surgery; therefore, we desperately wonder whether the enhanced osteogenesis help to prevent LBP.

The sensory innervation in degenerated endplates and upregulation of pain-receptor or cation channels are strongly linked with the nociceptive signaling of LBP.\textsuperscript{41,42} When endplates lesions occur, osteoclastic cells secrete Netrin-1 to induce CGRP+ sensory fibers extrusion and innervation in the spaces generated by osteoclast resorption.\textsuperscript{43} The presence of inflammatory mediators within endplates may accelerate disk degeneration and contribute to pathological nerve fiber ingrowth and sensory nerve sensitization.\textsuperscript{44,45} However, how to effectively disrupt pain-related nerve targets or inflammation remains largely unknown. After the endplates porosities were efficiently blocked by resveratrol, we observed the following series of thrilling phenomena. First, we found that abundant painful CGRP+ nerve fibers surrounding exposed or innervated into porous endplates at 8 weeks post-LSI surgery, which were appreciably eliminated by resveratrol. Likewise, a notable reduction of CGRP+ DRG neurons were observed by immunostaining in the resveratrol group at 8 weeks post-LSI surgery. Second, the inflammatory mediators PGE2 and COX2 levels in the endplates were elevated after 8 weeks LSI surgery, which were markedly reversed by resveratrol. Similarly, resveratrol efficiently reduced the LSI surgery-induced upregulation of TNFα and IL-1β in the endplates. Consistently, markedly enhanced hyperalgesia was recorded at 8 weeks post-LSI surgery, and a striking reduction in hyperalgesia was observed after resveratrol treatment. These discoveries indicated that resveratrol exert a superior role in blocking pain nerve fibers innervated into endplates, endplates innervation, and ultimately preventing LBP.

Under various internal and external conditions, the enhanced expression of SIRT1 in mesenchymal stem cells promotes osteogenic differentiation\textsuperscript{17} and protects against bone loss.\textsuperscript{18} Correspondingly, the downregulation of SIRT1 promotes osteoclastogenesis and osteolysis in mice.\textsuperscript{46,47} To verify whether resveratrol-mediated analgesic effect through the activation of SIRT1-triggered osteogenesis in the porous endplates, the \textit{SIRT1}_\textit{oxs}^+/− mice were employed. We observed that resveratrol-treated \textit{SIRT1}_\textit{wt} mice only showed few porosities in the endplates after LSI surgery, whereas resveratrol failed to prevent the development of endplates porosities in the \textit{SIRT1}_\textit{oxs}^−/− mice. The osterix was obvious detected in the \textit{SIRT1}_\textit{wt} mice following resveratrol treated after LSI surgery, while osterix expression was hardly observed in the porous endplates in \textit{SIRT1}_\textit{oxs}^+/− mice even though application of resveratrol. Moreover, in \textit{SIRT1}_\textit{wt} mice resveratrol dramatically increased the expression of Aggrecan and Runx2 after LSI surgery, and this effect was abrogated in \textit{SIRT1}_\textit{oxs}^−/− mice. The catabolic factor MMP13 expression in the endplates showed the opposite tendency to Aggrecan. These results demonstrated that resveratrol-mediated osteogenesis in the porous endplates through activation of SIRT1 in endplates osteoprogenitor cells.

Exactly as we expected, compared with the sham and resveratrol-treated \textit{SIRT1}_\textit{wt} mice, resveratrol failed to alleviate LSI surgery-induced LBP in \textit{SIRT1}_\textit{oxs}^+/− mice. We also discovered that the CGRP+ sensory nerves in the endplates were obviously depleted in the resveratrol-treated \textit{SIRT1}_\textit{wt} mice after LSI surgery, while the endplates in \textit{SIRT1}_\textit{oxs}^+/− mice still exhibited abundant CGRP+ sensory nerves. The results displayed that \textit{SIRT1}_\textit{oxs}+/− mice abrogated the effect of resveratrol played in blocking sensory nerves innervation in the endplates. Likewise, compared with \textit{SIRT1}_\textit{wt}, the Slit-3 expression in the endplates of the \textit{SIRT1}_\textit{oxs}^+/− mice significantly decreased after resveratrol treated. Taken together, resveratrol-mediated activation of SIRT1 in endplates osteoprogenitor cells contributed to the analgesic effect after LSI surgery. Hence, the treatment strategies which aim to promote the osteogenes in porous endplates, shows an extremely broad prospect in the prevention and treatment of LBP.

The guidelines previously recommended simple analgesics (eg, paracetamol) as part of the first-line care for LBP, however, recent evidence exhibits that paracetamol is no more effective than placebo for treatment of LBP.\textsuperscript{48} Advice, reassurance, encouragement of physical activity, and psychotherapy have gradually become recommended as first-line care for LBP patients.\textsuperscript{49,50} Among all above nondrug treatment measures, relief of anxiety to alleviate LBP has recently received progressive attention.\textsuperscript{51,52} Although there may be some controversy, recent studies indicate that SIRT1 plays important roles in the pathophysiology and treatment of anxiety.\textsuperscript{53-55} Brain-specific SIRT1 knockout mice showed a reduced anxiety and stress-resilient phenotype,\textsuperscript{55} whereas global SIRT1 overexpression mice showed increased anxiety and vulnerability to stress.\textsuperscript{54} Meanwhile, overexpressed SIRT1 in the brain does not affect anxiety-like behavior.\textsuperscript{55} The SIRT1-mediated control of anxiety may depend on the cell type, genetic background, and brain region. Interestingly, resveratrol successfully abolished the anxiety behaviors at 8 weeks post-LSI surgery. In past decades, converging evidence indicated that hippocampal inflammation is central to the development of anxiety symptoms in some cases.\textsuperscript{56-59} Clinical evidence reported that patients with anxiety disorder exhibit increased concentration of inflammatory cytokines in hippocampus.\textsuperscript{56} Animal models demonstrated that hippocampal inflammation mediates the development of anxiety-like behavior in response to stress stimuli.\textsuperscript{57} As expected, the gene levels of TNFα, IL-1β, and IL-6 in the hippocampus were appreciably elevated after LSI surgery, which were markedly attenuated by resveratrol. Aside from neuroinflammation, a reduction in the pCREB activation and BDNF expression in
the hippocampus also appears to intensify anxiety-like behaviors. Strategies to increase pCREB activity and BDNF expression are expected to provide new approaches for suppression of anxiety. As expected, immunostaining exhibited that pCREB activation and BDNF expression decreased after LSI surgery, and resveratrol appreciably restored the declined trend of pCREB and BDNF. Taken together, these results suggested that resveratrol efficiently alleviates hippocampal inflammation and restores pCREB activation, BDNF expression in hippocampus after LSI surgery.

5 | CONCLUSION

LSI surgery induced the porous endplates formation and then, led to sensory innervation and inflammation in endplates as well as neuroinflammation in the hippocampus, eventually leading to LBP and anxiety-like behavior in mice. The treatment strategy of elevation SIRT1 activation by resveratrol to enhance osteogenesis in porous endplates prove beneficial in blocking sensory innervation and inflammation in endplates, accompanied with the reduced neuroinflammation in hippocampus, therefore, efficiently alleviate LSI surgery-induced LBP and anxiety.

6 | Study Approval

All the animal experiments were performed in accordance with HUST policies on the use of laboratory animals. Experimental protocols were approved by the Animal Care and Use Committee of HUST.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflict of interest in connection with this article.

AUTHOR CONTRIBUTIONS

X. Lv, S. Chen, H. Liu, and Z. Shao designed the research. X. Lv, F. Gao, and B. Hu performed the experiments. S. Ni, Y. Wang, H. Kou, and Z. Song acquired and analyzed the data. X. Lv, S. Chen, X. Qing, and S. Wang conceived the study and wrote the manuscript. The integrity of this work is guaranteed by Dr. X. Lv and Dr. Z. Shao.

ORCID

Xiao Lv https://orcid.org/0000-0002-0175-5240

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