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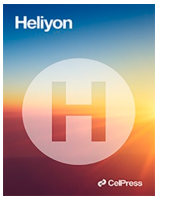

Research article

Resveratrol ameliorates pathological fibrosis of the myodural bridge by regulating the SIRT3/TGF-β1/Smad pathway

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ABSTRACT

Purpose: Pathological fibrosis of the myodural bridge (MDB) affects cerebrospinal fluid circulation. However, no optimal drug treatments are available. We aimed to explore the antifibrotic effect of resveratrol on bleomycin-induced pathological fibrosis of the MDB and its underlying mechanisms.

Methods: Genes common to the potential targets of resveratrol were determined using network pharmacology, genes related to muscle and tendon fibrosis were acquired from the GeneCards database, and genes related to MDB development were determined using Venny. These genes were considered potential resveratrol treatment targets in bleomycin-induced pathological fibrosis of the MDB and were annotated using bioinformatics methods. We validated the intersected genes using quantitative real-time polymerase chain reaction (qRT-PCR) and performed molecular docking analysis to calculate the binding activity between the target gene and resveratrol. Hematoxylin and eosin and Masson staining were used to detect the morphological changes in bleomycin-induced fibrosis of the MDB following resveratrol treatment. We used qRT-PCR and immunohistochemistry to evaluate the expression of the sirtuin 3 (SIRT3)/transforming growth factor-β1 (TGF-β1)/Smad pathway and the profibrotic markers α-smooth muscle actin (α-SMA) and Collagen I.

Results: Through network pharmacology and bioinformatics analyses, we identified four core intersected genes, and SIRT3 expression was validated using qRT-PCR. Molecular docking analysis revealed that resveratrol had good binding affinity for SIRT3. Resveratrol ameliorated morphological abnormalities in bleomycin-induced pathological fibrosis of the MDB by inhibiting fibroblast activation and excessive collagen fiber deposition. Resveratrol exerted its antifibrotic effect by regulating the SIRT3/TGF-β1/Smad pathway.

Conclusion: Resveratrol has an antifibrotic effect in bleomycin-induced pathological fibrosis of the MDB *in vivo* and may be considered a novel therapeutic strategy.

1. Introduction

The myodural bridge (MDB) is a highly conserved physiological structure in mammals, which connects the suboccipital muscles to

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the cervical spinal dura mater (SDM) and transmits tensile forces generated by the suboccipital muscles to the SDM [1,2]. The MDB promotes to the dynamics of cerebrospinal fluid movement substantially [3–5]. Pathological fibrosis of the MDB presents with increased stiffening and decreased compliance, which alters cerebrospinal fluid circulation over time [6,7]. Clinically, for patients with chronic headache who experienced treatment failure using conventional anti-headache drugs, surgical separation of the tissue connecting the suboccipital muscles and SDM, in a procedure termed "MDB release," effectively relieves the abnormal dural tone and ameliorates headache [8–10]. In our previous study, we reported that bleomycin injections mimic MDB pathological fibrosis and induce headache in animal models [6]. However, the pathogenesis of MDB fibrosis remains unclear, and no optimal drug treatment is currently available. Therefore, the identification of new agents for the treatment of MDB pathological fibrosis is crucial.

Resveratrol is a naturally occurring phytoalexin, produced by some spermatophytes, which possesses various biological functions, including antioxidant, anti-inflammatory, antifibrotic, and cardioprotective effects [11–13]. Resveratrol exerts its antifibrotic effect by inhibiting fibroblast activation and reducing excessive collagen deposition through downregulating the transforming growth factor-β1 (TGF-β1) pathway in several tissue types [14–16]. However, the mechanism of action of resveratrol in bleomycin-induced MDB fibrosis remains unknown. Therefore, in the present study, we aimed to provide a theoretical basis for the clinical application of resveratrol in the treatment of MDB pathological fibrosis.

2. Materials and methods

2.1. Network pharmacology

PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was searched using the term "resveratrol" to acquire its SMILE string, and SwissADME (http://www.swissadme.ch/) was utilized to explore the pharmacokinetic properties of resveratrol. All resveratrol targets were identified using Herb (http://herb.ac.cn). Genes related to muscle and tendon fibrosis were searched in GeneCards database (https://www.genecards.org/). Genes common to the list of resveratrol targets, genes related to muscle and tendon fibrosis, and differentially expressed genes (DEGs) related to MDB development acquired from our previous study were identified with Venny (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.2. Bioinformatics analysis

Intersected genes were annotated using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, and statistical significance was set at an adjusted P-value <0.05. A protein-protein interaction network was built using Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org).

2.3. Animal model

All animal treatments were performed with permission from the Chinese Authorities for Animal Protection and approved by the Ethics Committee of Dalian Medical University (approval number: AEE21085). Healthy 10-week-old male Institute of Cancer Research (ICR) mice (Changsheng, China) were anesthetized with tribromoethanol (500 mg/kg) and randomly divided into five groups: the control (without any treatment), sham (injected with phosphate-buffered saline only), experimental (injected with bleomycin), low-dose resveratrol-treated (injected with bleomycin +5 mg/kg/d resveratrol), and high-dose resveratrol-treated groups (injected with bleomycin +20 mg/kg/d resveratrol). The detailed modeling method was described in our previous study [6]. Briefly, a single injection of bleomycin (25 μL, 40 mg/mL) was administrated into the posterior atlanto-occipital interspace of ICR mice. The mice were euthanized after four weeks, and the MDB was collected.

2.4. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using a total RNA isolation kit (Vazyme, China). Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized from each RNA sample using HiScript II Q RT SuperMix (Vazyme). qRT-PCR was performed using SYBR qPCR Master Mix (Vazyme), and the target gene expression levels were quantified relative to the housekeeping gene GAPDH using the $2^{-\Delta\Delta CT}$ method. The primer sequences used are listed in Supplementary Material 1.

2.5. Molecular docking

Molecular docking analysis of sirtuin 3 (SIRT3) and resveratrol was performed using AutoDock Vina. The three-dimensional structure of SIRT3 was acquired from Protein Data Bank (http://www.rcsb.org). Water molecules and original ligands were removed from SIRT3 before hydrogenation, charge calculations, and nonpolar hydrogen combinations using AutoDock Tools.

2.6. Histological staining

Hematoxylin and eosin (HE) and Masson staining were performed to observe morphological changes. Following conventional perfusion, the tissue samples were fixed with 4 % paraformaldehyde. After regular paraffin embedding, 6-μm-thick sections were acquired. Detailed procedures were performed according to the manufacturer's instructions (Solarbio, China). Images were captured

using an optical microscope (Nikon, Japan).

2.7. Immunohistochemistry (IHC)

Primary antibodies specific to SIRT3 (1:50; Cell Signaling Technology, USA), TGF-β1 (1:50; Proteintech, China), Smad2/3 (1:100;

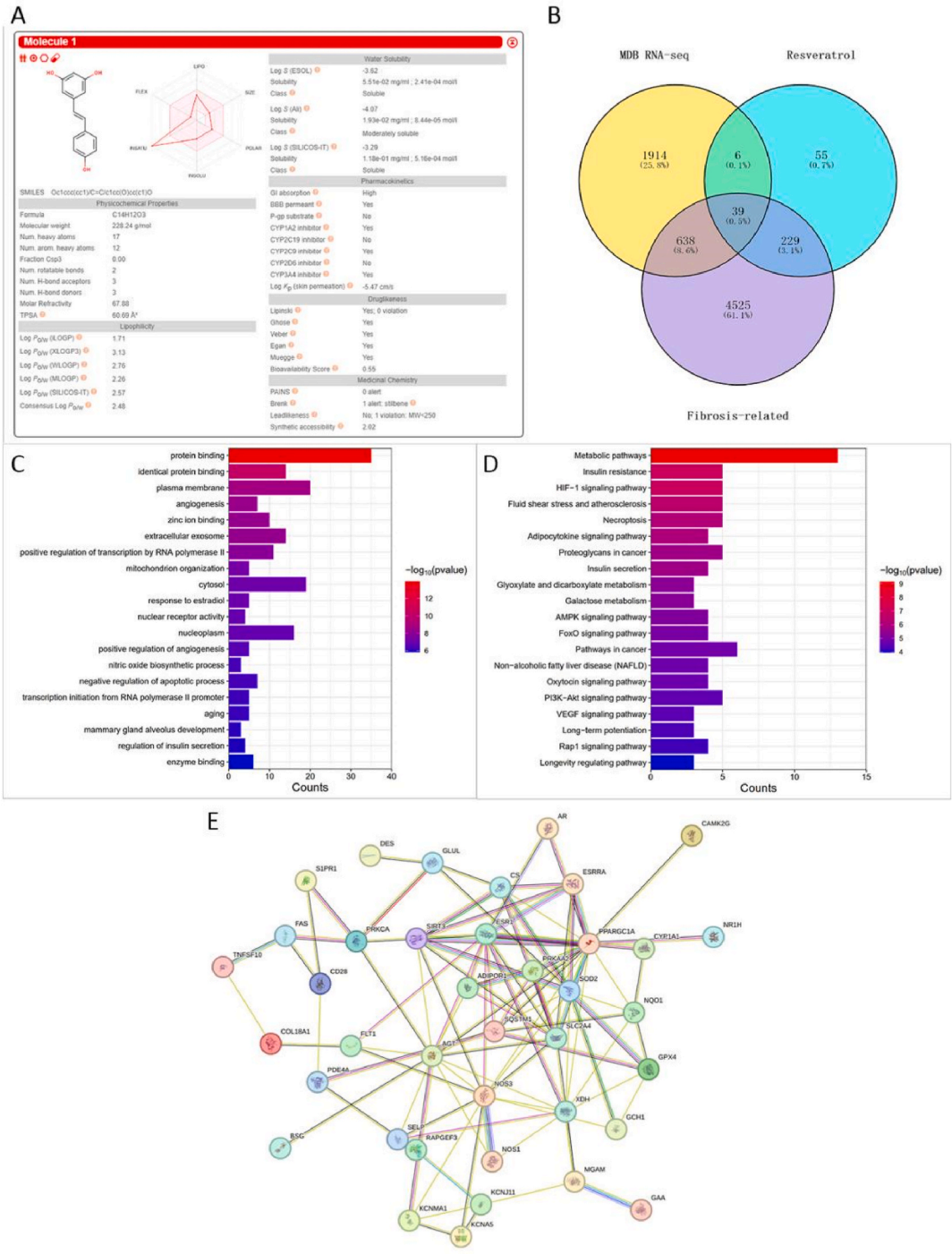

Fig. 1. Network pharmacology and bioinformatics analysis of resveratrol in the treatment of bleomycin-induced MDB pathological fibrosis. (A) The pharmacokinetic properties of resveratrol in the SwissADME platform. (B) Venn of the intersected genes. (C) GO, (D) KEGG, and (E) protein-protein interaction network of the intersected genes. MDB, myodural bridge; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Cell Signaling Technology), Phospho Smad2/3 (1:100; Cell Signaling Technology), alpha smooth muscle actin (α-SMA) (1:200; Abcam, USA), and Collagen I (1:200; Abcam) were incubated with the sections after routine protocol.

2.8. Statistical analysis

Unpaired two-tailed Student's t-tests were used to analyze the statistical significance between two groups. GraphPad Prism 8 (GraphPad Software, USA) was used for statistical analysis and visualization. ImageJ software (National Institutes of Health, Bethesda,

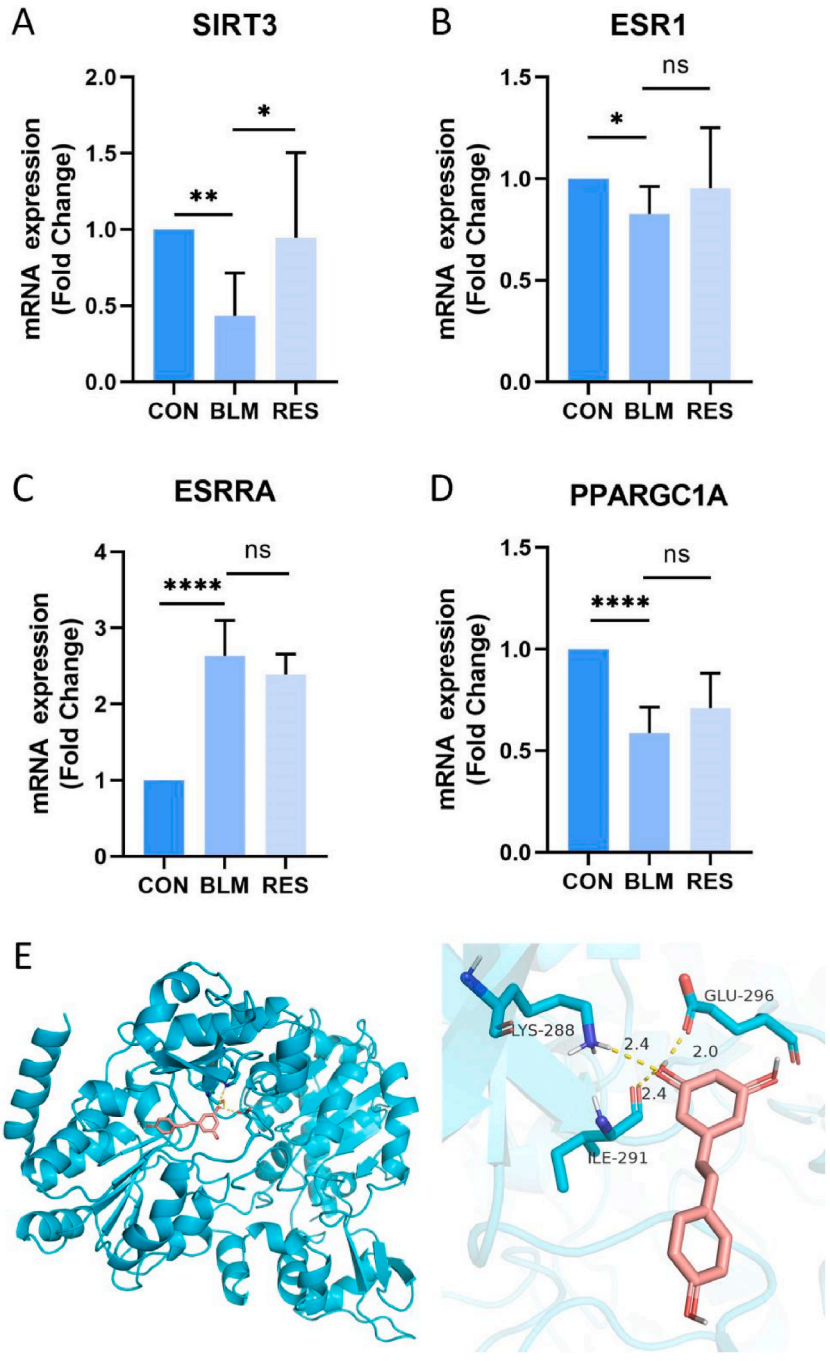

Fig. 2. Validation of SIRT3 expression in bleomycin-induced MDB pathological fibrosis and molecular docking analysis. (A–D) mRNA expression of the four core intersected genes, SIRT3, ESR1, ESRRA, and PPARGC1A (n = 5/group). (E) Molecular docking analysis of resveratrol with potential target protein SIRT3. MDB, myodural bridge. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

USA) was used to analyze the images. Five replicates were performed for all experiments, and the results are presented as the mean ± SEM. Statistical significance was set at $P < 0.05$, and ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

3. Results

3.1. Network pharmacology and bioinformatics analysis

The pharmacokinetic properties of resveratrol are presented in Supplementary Material 2. The positive results of Lipinski's

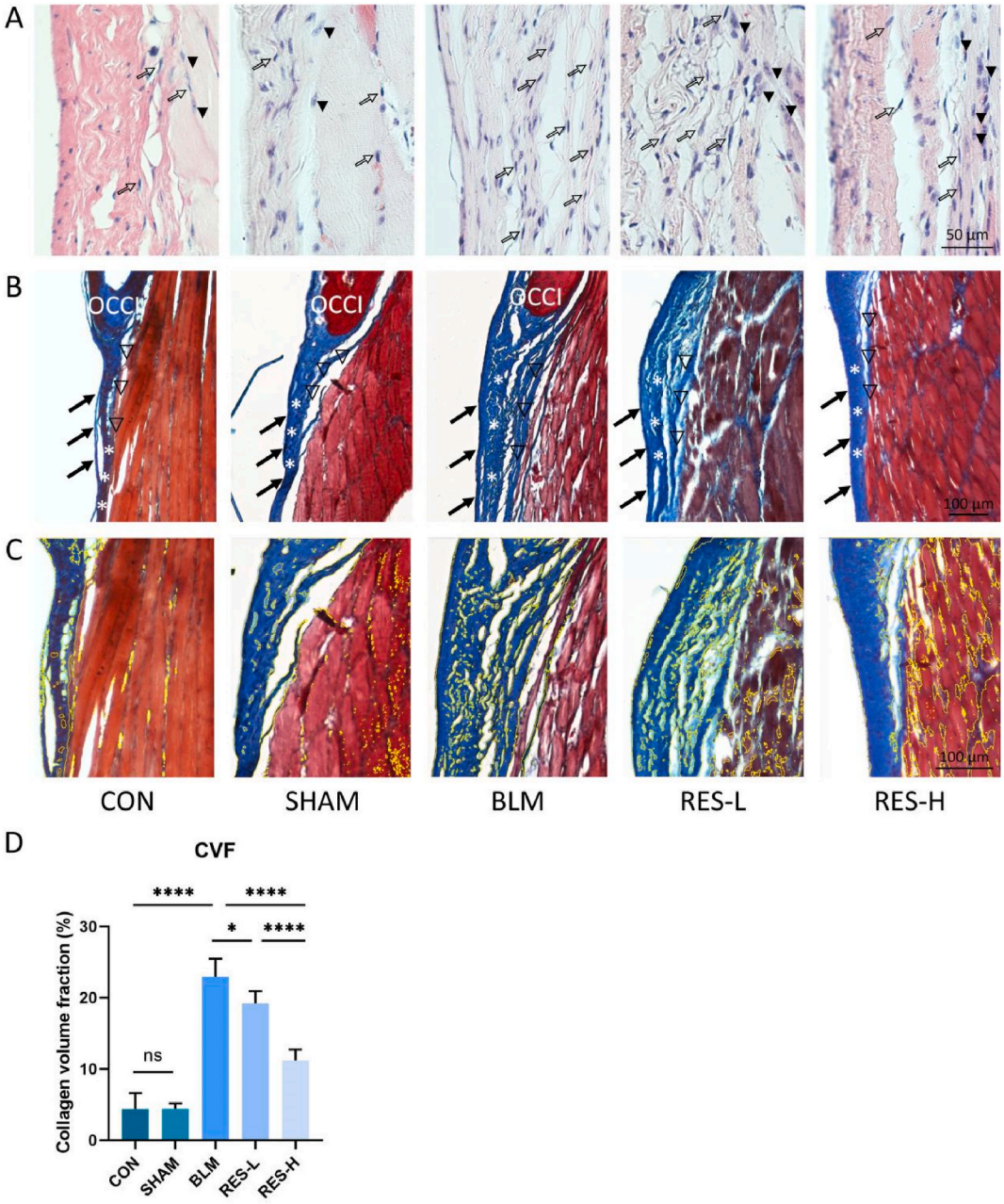

Fig. 3. Resveratrol ameliorated the morphological abnormalities in bleomycin-induced MDB pathological fibrosis. (A–B) HE and Masson staining in the control, sham, experimental, low-dose resveratrol-treated, and high-dose resveratrol-treated groups (n = 5/group). (C–D) Quantitative analysis of collagen volume fraction of MDB fibers (n = 5/group). HE, hematoxylin and eosin; MDB, myodural bridge. white arrows, fibroblast cell; ▼, myonuclear cell; OCCI, occipital bone; black arrows, spinal dura mater; *, posterior atlanto-occipital membrane▽, MDB fibers; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

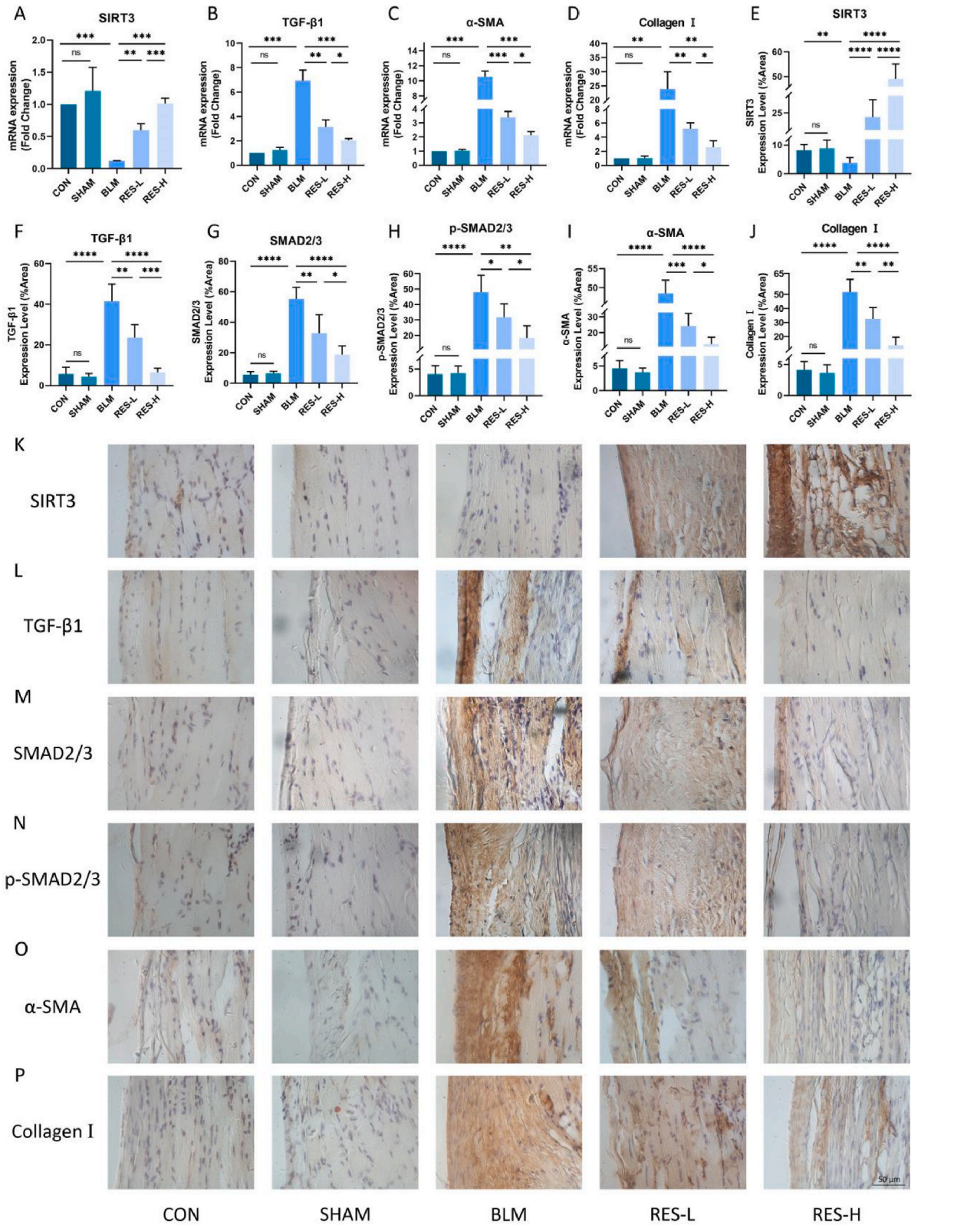

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Fig. 4. Resveratrol regulated the SIRT3/TGF-β1/Smad pathway to abrogate bleomycin-induced MDB pathological fibrosis. (A–D) mRNA expression of SIRT3, TGF-β1, α-SMA, and Collagen I in each group (n = 5/group). (E-H, K–N) Protein expression of SIRT3, TGF-β1, Smad2/3 and p-Smad2/3 in each group (n = 5/group). (I, J, O, P) Protein expression of profibrotic markers α-SMA and Collagen I in each group (n = 5/group). MDB, myodural bridge; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

evaluation revealed that resveratrol has good pharmacokinetic properties, high bioavailability *in vivo* (Fig. 1A). Using the Venny platform, 39 genes common to 326 potential targets of resveratrol, 5343 genes related to muscle and tendon fibrosis, and 5545 DEGs related to MDB development were identified. These 39 intersected genes were considered potential targets of resveratrol in the bleomycin-induced MDB pathological fibrosis treatment (Fig. 1B–Supplementary Material 3). The GO analysis indicated that the intersected genes were enriched in mitochondrial tissues and were involved in aging and apoptosis regulation (Fig. 1C). The KEGG analysis indicated that the intersected genes were enriched in metabolism-related pathways, the PI3K-Akt signaling pathway, and the AMPK signaling pathway (Fig. 1D). The confidence level was set to "0.98″ to hide free nodes, and four core intersected genes were obtained, including SIRT3, ESR1, ESRRA, and PPARGC1A, in the protein-protein interaction network (Fig. 1E).

3.2. Validation of SIRT3 expression and molecular docking analysis

We conducted qRT-PCR to validate the expression of four core intersected genes: SIRT3, ESR1, ESRRA, and PPARGC1A. Only SIRT3 mRNA was significantly decreased in the experimental group and significantly increased after high-dose resveratrol treatment (Fig. 2A–D). As there is no unified standard of the binding free energy at present, we selected energy ≤ -5.0 kcal/mol as the evaluation criterion, according to a previous study [17]. Molecular docking indicated that resveratrol and SIRT3 had a strong binding activity, with binding free energy −7.4 (Fig. 2E–Supplementary Material 4). Therefore, SIRT3 was selected for further analysis.

3.3. Resveratrol ameliorated the morphological abnormalities in bleomycin-induced MDB pathological fibrosis

Pathological fibrosis is characterized by fibroblast activation and over-deposition of collagen fibers [18]. Analysis of HE staining revealed that the number of fibroblast nuclei in the MDB region was higher in the experimental group than that in the control and sham groups. After resveratrol treatment, the number of fibroblast nuclei decreased (Fig. 3A). Masson staining showed that the orientations of MDB fibers among the different groups were similar, connecting the suboccipital musculature to the SDM. However, the number of MDB fibers increased in the experimental group, and the fibers were more densely arranged than those in the control and sham groups. Resveratrol treatment decreased the number of fibers, and the fibers appeared loosely arranged (Fig. 3B). For quantitative analysis, we calculated the collagen volume fraction of MDB fibers (Fig. 3C). The effect of resveratrol was dose-dependent (Fig. 3D). Resveratrol treatment ameliorated the morphological abnormalities observed in bleomycin-induced MCB fibrosis, such as fibroblast activation and overdeposition of collagen fibers.

3.4. Resveratrol regulated the SIRT3/TGF-β1/Smad pathway to abrogate fibrosis

We evaluated the expression of the SIRT3/TGF-β1/Smad pathway and the profibrotic markers α-SMA and Collagen I. SIRT3 was decreased in the experimental group compared with that in the control and sham groups; however, this decrease was reversed by resveratrol in a dose-dependent manner at the mRNA expression level (Fig. 4A). The mRNA expression levels of TGF-β1, α-SMA, and Collagen I were increased in the experimental group compared with those in the control and sham groups; however, these increases were dose-dependently inhibited by resveratrol (Fig. 4B–D). Consistent with the qRT-PCR results, the protein expression levels of SIRT3/TGF-β1/Smad pathway were regulated by resveratrol in a concentration-dependent manner (Fig. 4E–H, K-N). To detect levels of Smad2/3, we examined its phosphorylated form. The protein expression levels of α-SMA and Collagen I detected using immunohistochemistry staining were consistent with those detected via qRT-PCR (Fig. 4I, J, O, P). Resveratrol increased levels of SIRT3, while suppressing the expression of the TGF-β1/Smad pathway.

4. Discussion

The ubiquitous presence of the MDB in mammals suggests that it may be an evolutionarily conserved anatomical structure that has an important functional role, such as in transmitting tensional forces produced by suboccipital muscle contraction to the upper cervical SDM during head movements [1,19]. This transformation leads to an alteration in the subdural and subarachnoid volumes, which may produce negative pressure and influence the dynamic circulation of cerebrospinal fluid [4,7,20]. Clinically, it has been well documented that the pathological fibrosis of MDB would fail to maintain and transmit constant tension, leading to a weakened promotion effect on the flow of cerebrospinal fluid and a series of abnormal clinical manifestations [21,22]. Symptomatic adult Chiari malformation Type I presents with a mechanical overload of the MDB and further causes a functionally abnormal MDB consistent with this pathological change [23]. However, the exact pathogenesis of MDB pathological fibrosis is poorly understood, and the only treatment strategy is surgical separation of the tissue connecting the suboccipital muscles and SDM, called "MDB release" [8–10]. No optimal drug treatment is currently available to alleviate cervical headache induced by MDB pathological fibrosis. Therefore, the development of treatments is urgently required.

Bleomycin injection induces a fibrotic response in the MDB, characterized by increased dural stiffness and decreased MDB

compliance, which can cause cervical headache, as reported in our previous *in vivo* study [6,22]. Therefore, we utilized the same method in the present study and yielded consistent results. The pathogenesis of fibrosis is complicated, and it has been widely confirmed that the proliferation and differentiation of fibroblasts are important aspects [24,25]. The TGF-β1-mediated fibroblast activation secretes profibrotic mediators and induces extracellular matrix deposition, including collagen, fibronectin, and laminin, which are deposited in tissues to replace normal structures [26–28]. Therefore, controlling fibroblast activation is of great importance for the treatment of bleomycin-induced MDB pathological fibrosis. In this study, we found that resveratrol significantly ameliorated morphological abnormalities in MDB pathological fibrosis, reducing fibroblast activation and collagen fiber deposition, as shown by HE and Masson staining, respectively.

Pharmacokinetic properties, such as absorption, distribution, metabolism, and excretion, helps identify candidates with good pharmacokinetic characteristics and avoids costly failures [29,30]. Based on network pharmacology and bioinformatics analyses, we gained insight into the underlying targets of resveratrol in MDB pathological fibrosis. Four core intersected genes were confirmed by performing qRT-PCR. Molecular docking analysis revealed that resveratrol has a strong affinity for SIRT3 [31]. SIRT3 downregulation was reported to be participated in the pathological process, while the activation of SIRT3 inhibited fibroblast activation and excessive deposition of collagen fibers via inhibition of the TGF-β1/Smad3 pathway [32–34]. However, TGF-β1, as a powerful inducer of tissue fibrosis, could lead to the consumption of endogenous SIRT3 [28,35]. Furthermore, SIRT3 overexpression may have therapeutic potential for reversing pathological fibrosis through decreasing fibroblast activation [36,37]. Therefore, the SIRT3/TGF-β1/Smad pathway is crucial in the development of pathological fibrosis.

To further explore the antifibrotic mechanism of resveratrol in bleomycin-induced MDB pathological fibrosis, we focused on SIRT3/TGF-β1/Smad pathway factor expression. TGF-β1 is considered the most critical fibrotic factor, inducing fibroblast activation, the synthesis of collagen to form the extracellular matrix, and the transcription of fibrosis-related genes encoding proteins such as Collagen I and α-SMA [38]. TGF-β pathway is activated after the binding of TGF-β to TGF-β type II receptor. This binding enhances activation of the TGF-β type I receptor, which contains a kinase domain phosphorylating Smad2/3 [39,40]. Phosphorylated Smad2/3 combines with Smad4 to form a complex that translocates into the nucleus and regulates gene expression. This complex is crucial for both transmitting TGF-β1-mediated signals to the nucleus and promoting profibrotic genes expression while inhibiting Smad7 expression, leading to the α-SMA overexpression, which further activates TGF-β1 [41,42]. Collectively, TGF-β1/Smad pathway dysfunction is a critical pathogenic mechanism of tissue fibrosis [43]. Consistent with previous studies, we found that resveratrol increased SIRT3 levels and inhibited the TGF-β1/Smad pathway [28,44]. The antifibrotic action of resveratrol was presented by increasing SIRT3 levels while suppressing TGF-β1/Smad signaling *in vivo*.

Resveratrol is a naturally occurring polyphenol, abundant in grapes, known for its potent antifibrotic property [45]. Several studies have demonstrated that resveratrol has effectiveness in treating fibrosis-related diseases as it mitigates the condition by reducing inflammation and oxidative stress level [46,47]. Therefore, these studies offer compelling evidence supporting the feasibility of using resveratrol in the pathological fibrosis of MDB treatment. This is the first study to report on the connection between pathological fibrosis of MDB and resveratrol, as well as the underlying mechanism. Our findings suggest that resveratrol diminishes fibrosis in pathological fibrosis of MDB by reinstating fibrosis through the SIRT3/TGF-β1/Smad pathway. Therefore, clinicians should monitor MDB pathological fibrosis in cervical headache patients via magnetic resonance imaging, and resveratrol supplement, as a non-invasive treatment method, could be considered in future clinical practice [48,49].

This study has some limitations. Specific inhibitors or adenovirus-associated vectors of SIRT3 should be used *in vivo* to further validate the functions of SIRT3. A more convincing method would be to verify these results in SIRT3 knockout mice. However, no ideal strategy has been developed to precisely knock down or knock out SIRT3 in the MDB, which could mitigate its effects on other parts of the body in mice. Therefore, more rigorous experimental methods to validate these effects are needed.

5. Conclusion

We explored the antifibrotic effect of resveratrol on pathological fibrosis of MDB and the underlying mechanisms involved. Resveratrol ameliorated morphological abnormalities such as fibroblast activation and over-deposition of collagen fibers in bleomycin-induced MDB pathological fibrosis. Mechanically, resveratrol increased levels of SIRT3 while suppressing TGF-β1/Smad signaling. In conclusion, we provided a theoretical basis for the clinical application of resveratrol in the treatment of MDB pathological fibrosis.

Data availability statement

Data will be made available on request.

Funding

No funding was received.

CRediT authorship contribution statement

Tao Qin: Writing – original draft, Conceptualization. **Xue Song:** Formal analysis, Data curation. **Qing Shao:** Formal analysis, Data curation. **Jianfei Zhang:** Writing – review & editing. **Hongjin Sui:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34974.

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