Suberoylanilide hydroxamic acid attenuates epidural fibrosis *via* inhibiting myofibroblast differentiation and increasing fibroblast apoptosis

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Abstract. – OBJECTIVE: Epidural fibrosis represents a fatal stage of failed back surgery syndrome (FBSS) of known and idiopathic etiology, but no valid therapy is presently available. Previous evidence demonstrated that suberoylanilide hydroxamic acid (SAHA), a histone deacetylases inhibitor, has antifibrotic and anti-inflammatory potential. Current studies have proved that SAHA inhibits myofibroblast differentiation and increases fibroblast apoptosis to attenuate epidural fibrosis. The purpose of this study was to investigate the effect and mechanism of SAHA on repressing epidural fibrosis.

PATIENTS AND METHODS: First, the levels of acetylation of histone and α-tubulin in adult human fibroblasts (AHF) and human epidural fibroblasts (HEF) were analyzed following SAHA and transforming growth factor-β (TGF-β) treatment. Then, mRNA and protein obtained from human fibroblasts following TGF-β activation and SAHA treatment *in vitro* culture were used to test the influence of SAHA on the activation and apoptosis of fibroblasts, so as to further explore the related mechanism of SAHA. Then, a laminectomy model was established in rats to observe the therapeutic effect of SAHA on epidural scar tissue.

RESULTS: The present research proved that the increases of HDAC 3 and α-tubulin were observed in AHF and HEF after TGF-β administration, but SAHA decreased HDAC 3 and α-tubulin expressions. In addition, cell study demonstrated that SAHA inhibited fibroblast activation *via* decreasing TGF-β function and accelerated apoptosis by promoting cleaved-caspase-3. In the epidural fibrosis model, it was found that SAHA weakened scar hyperplasia and collagen deposition, and effectively inhibited the process of epidural fibrosis.

CONCLUSIONS: These results indicated that SAHA inhibited HDAC 3 expression, decreased TGF-β effect, and enhanced caspase-3 in fibroblasts, leading reduction of myofibroblast activation and apoptosis elevation. Hence, SAHA ameliorated epidural fibrosis development.

Key Words:
Antifibrotic, Collagen, Fibroblast, Histone deacetylase inhibitor, Suberoylanilide hydroxamic acid, Epidural fibrosis.

Introduction

Failed back surgery syndrome (FBSS) constitutes a diverse set of spinal cord and nerve roots disorders following surgery with different levels of pain and numbness resulting in neurologic loss of function and even dysfunction[1,2]. The most common representative of FBSS is epidural fibrosis. The general pathological feature of epidural fibrosis is the excessive collagen deposition which destroys the architecture of the normal epidural tissue[3]. The current therapy for epidural fibrosis is based on second operation to relieve the symptoms of nerve compression[4,5]. However, this treatment cannot fundamentally solve the pathogenesis of epidural fibrosis, which critically results from the activated state of fibroblasts/myofibroblasts[6,7]. Clearly, epidural fibrosis remains a huge therapeutic challenge and there is an urgent need to develop effective anti-fibrotic drugs. Histone deacetylase inhib-

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itors (HDACIs), as a new class of anti-tumor drugs, can inhibit tumor cell proliferation[8], induce cell cycle arrest[9], promote cell differentiation or apoptosis[10] after acting on tumor cells by inhibiting the activity of histone deacetylases. HDACIs might also inhibit collagen production in myocardial remodeling and pulmonary fibrosis[11,12]. Phenylbutyrate has been reported[13] to decrease TGF-β-stimulated collagen I mRNA and protein levels in the lung fibroblast. Valproic acid alleviated Ang II-induced cardiac fibrosis and myocardial pericytes by inhibiting HDAC 4-dependent phosphorylation of ERK[14]. In addition, Trichostatin A inhibited collagen production in sclerosing fibroblasts throughout the body and reduced the total amount of collagen deposition in bleomycin-induced skin fibrosis in mice[15]. Therefore, suberoylanilide hydroxamic acid (SAHA) was evaluated, and has been clinically used as a third-line drug to treat cutaneous T-cell lymphoma, and is being evaluated for other types of cancer[16,17]. Here, the anti-fibrosis potential of SAHA in human fibroblasts (AHF) and human epidural fibroblasts (HEF) treated with TGF-β administration, as well as its anti-fibrosis effect after fibroblasts *in vitro* and laminectomy *in vivo* was explored.

Patients and Methods

Cells and Drug Treatment

HEF was obtained from patients undergoing secondary decompression surgery and the procedure was approved by the Ethics Committee of Honghui Hospital. We also acquired informed consent from patients or their families prior to sample collection. A total of 15 patients (mean age, 43, age range, 38-54, 8 males, 7 females) donated their epidural scar tissues. Pending tissues were stored in liquid nitrogen until conducted the continuing study. AHF was purchased from the Chinese Academy of Medical Science. This study was approved by the Ethics Committee of the Honghui Hospital, Xi'an Jiaotong University, and signed written informed consents were obtained from all participants before the study.

Fresh epidural scar tissues were washed by Dulbecco's Modified Eagle's Medium and Ham's F-12 medium (DMEM/F12; Gibco, Rockville, MD, USA) and fragmentized. Dissociation was performed using 0.25% trypsin solution at 37°C for 15 min. Then, the mixture was treated with 0.15% type II collagenase at 37°C overnight, and the cell solution was transferred onto a cell strainer with 100 μm pore sizes and resuspended in DMEM/F12 containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). After that, all the cells were seeded in 5×5 cm^2 flask and TGF-β (10 ng/mL, sigma, St. Louis, MO, USA) was stimulated fibroblasts to evoke differentiation. Finally, SAHA (5 μM MedChemExpress, Monmouth Junction, NJ, USA) was used to treat cells.

Rats

Eight-week-old Sprague Dawley (SD) rats obtained from Xi'an Jiaotong University Animal Center, were bred and maintained at the Xi'an Jiaotong University. This study was approved by the Institutional Committee of Xi'an Jiaotong University, and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Xi'an Jiaotong University.

Laminectomy and Drug Injection

Rats received skin preparation following anesthesia with 10% chloral hydrate (4 mL/kg). After disinfection, a back incision was made to separate the fascia layer and the muscle layer and laminectomy was performed at T10. After rinsing by normal saline, the incision was closed and disinfected post hemostasis. Then, 10 mg/kg SAHA was administered once daily for 7 days *via* intraperitoneal injection.

Cell Viability Assay

Cell Counting Kit-8 (CCK-8) assay was performed to measure fibroblasts viability using CCK-8 Cell Viability/Cytotoxicity Assay Kit (C0009, Beyotime, Shanghai, China) following the manufacturer's protocol. Briefly, fibroblasts were transferred in 96-well plates with the density of 1×10^4 cells/well. Following TGF-β and SAHA absorbance was then measured using a microtiter plate reader (LabSystems Multiskan, Helsinki, Finland) at 570 nm.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA of fibroblasts or scar tissue were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) abided by the manufacturer's protocol. Complementary deoxyribose nucleic acid (cDNA) synthesis was conducted using the PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). Then, fibronectin, colla-

gen I, α-SMA, caspase3, bax-2, bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected using the SYBR PremixEx Taq II kit (RR820A, TaKaRa, Otsu, Shiga, Japan). The primers were listed as follows and the $2^{-\Delta\Delta Ct}$ methods was used to calculate the relative mRNA levels. Collagen I: Forward, 5′-CATCAAGGTCTTCTGCGACA-3′, Reverse, 5′-CTTGGGGTTCTTGCTGATGT-3′, *α-SMA:* Forward, 5′- GGCTCTGGGCTCTGTAAGG -3’, Reverse, 5’-CTCTTG CTCTGGGCTTCATC-3’, fibronectin: Forward, 5’-ACAACCCCTACAAACGGCCA-3’, Reverse, 5’-TAGTCAATGCCCGGCTCCAG-3’, caspase3: Forward, 5′-GCCATCGTGGCTAAACAGGTA-3′, Reverse, 5′-GTTGGTGTTCATCCGCTTGC-3′, bax-2: Forward, 5′-CTGACAGTTTTCTGACGG-3′, Reverse, 5′-TCAGCCACTTCCAGA-3′, bcl-2: Forward, 5′-GCTACCGTCGTGACTTCGC-3′, Reverse, 5′-CCCCACCGAACTCAAAGAAGG-3′, GAPDH: Forward, 5′-GCAAGTTCAACGGCACAG, Reverse, 5′-GCCAGTAGACTCCACGACCAT.

Western Blotting Analysis

Scar tissue or fibroblasts were treated using a Total Protein Extraction Kit (KeyGEN, Nanjing, China) with phosphatase and protease inhibitors. Following violent oscillation and low temperature centrifugation, the protein concentration was measured with the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and balanced. Then, the proteins were separated in10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After that, they were blocked with a Quick Block Solution (EpiZyme, Shanghai, China) and incubated overnight at low temperature with the primary antibodies: acetylated histone 3 (1:3000; Upstate Biotechnology, Lake Placid, NY, USA), acetylated a-tubulin (1:2500; Upstate Biotechnology, Lake Placid, NY, USA), anti-bax 2 (Abcam, Cambridge, MA, USA, 1:1000), anti-bcl 2 (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase 3 (Abcam, Cambridge, MA, USA, 1:1000), anti-collagen I (Millipore, Billerica, MA, USA, 1:1000), anti-α-SMA (Abcam, Cambridge, MA, USA, 1:1000), anti-fibronectin (Abcam, Cambridge, MA, USA, 1:500), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000). Following washing with Tris-Buffered Saline-Tween (TBST) and incubated with the secondary antibody (Abcam, Cambridge, MA, USA, 1:2000) at room temperature, the proteins were visualized and using the enhanced chemiluminescence system.

Flow Cytometry Analysis

Fibroblast apoptosis degree was measured using Apoptosis Detection Kit (KeyGEN, Nanjing, China). Following the manufacturer’s protocol, Annexin V-FITC and propidium iodide (PI) were stained with fibroblasts for 30 minutes. Then, the cells were sorted and analyzed using a fluorescence-activated cell sorting flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data in normality displayed by the means ± standard deviations. Comparisons of data between the two groups were analyzed using Student’s *t*-test, while comparisons among multiple groups were done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Statistical analysis was performed using GraphPad Prism (Version X, La Jolla, CA, USA). $p < 0.05$ represented statistical significance.

Results

SAHA Induces Hyperacetylation of Histone 3 and α-Tubulin in Fibroblast

First, AHF and HEF were treated with TGF-β and SAHA to observe the expression levels of acetylated histone 3 and acetylated α-tubulin in both cells. In AHF, decreased acetylated histone 3 and acetylated α-tubulin expressions were detected after SAHA treatment alone. However, after TGF-β treatment, acetylated histone 3 and acetylated α-tubulin expression in AHF were significantly increased compared with the control group. After co-treatment with TGF-β and SAHA, it was found that acetylated histone 3 and acetylated α-tubulin levels were significantly decreased compared with the TGF-β activated group. In addition, consistent trends were found in HEF, and acetylated histone 3 and acetylated α-tubulin expressions were significantly increased in HEF groups compared with those in AHF (Figure 1A-1C). Therefore, it was observed that HDAC3 and α-tubulin expressions were increased in AHF and HEF after TGF-β activation and SAHA mediated the acetylated histone 3 and α-tubulin expressions.

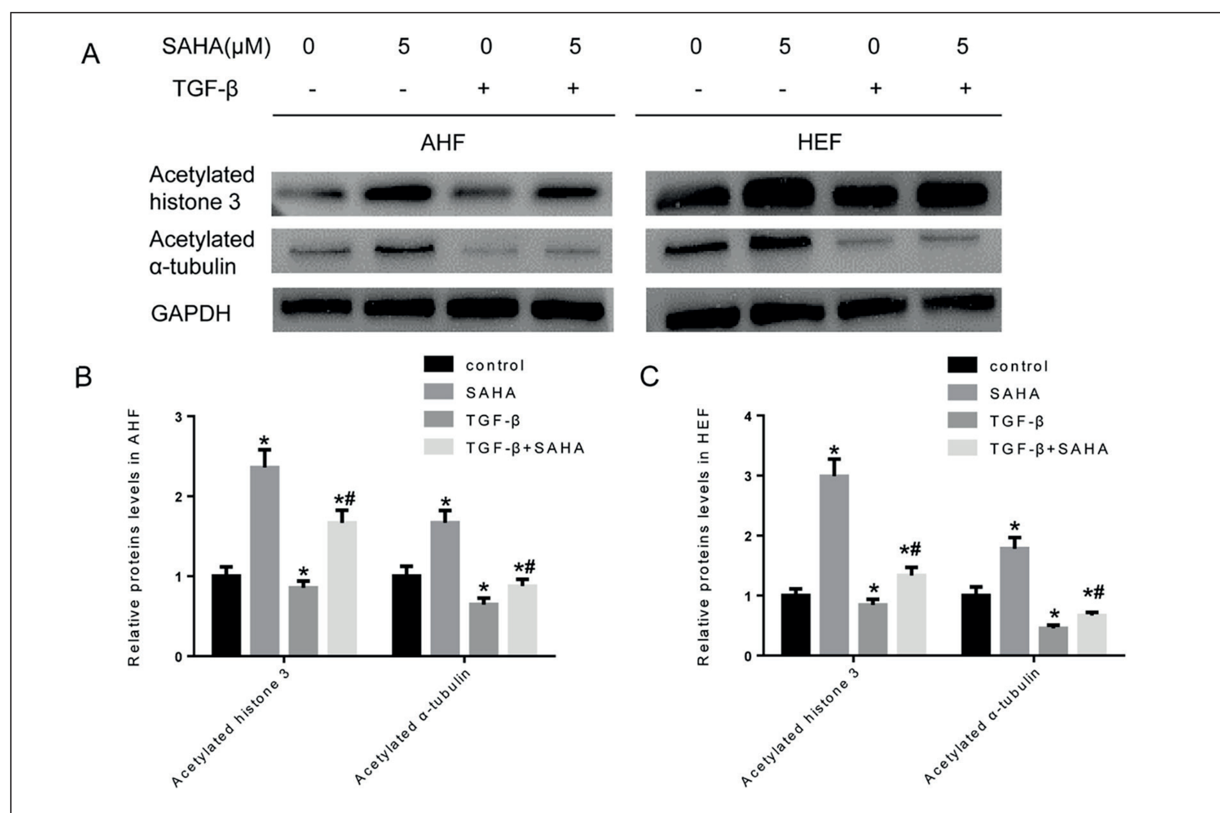

Figure 1. SAHA induces hyperacetylation of histone 3 and α-tubulin in fibroblast. **A,** Representative Western blotting of acetylated histone 3 and acetylated α-tubulin in the AHF and the HEF with SAHA (5 μM) and (or) TGF-β treatment. **B,** Representative protein analysis of acetylated histone 3 and acetylated α-tubulin in the AHF with SAHA and (or) TGF-β treatment. **C,** Representative protein analysis of acetylated histone 3 and acetylated α-tubulin in the HEF with SAHA and (or) TGF-β treatment. "*" means *vs.* control group with statistical significance. "#" means *vs.* TGF-β group with statistical significance.

SAHA Inhibits TGF-β-Induced Myofibroblast Transdifferentiation

Further, whether the utilization of SAHA affects the activation and transdifferentiation of fibroblast was investigated *via* TGF-β treatment *in vitro* culture of AHF. Studies have shown that TGF-β treatment could promote the activation of fibroblasts into myofibroblasts, which are highly expressed in α-smooth muscle actin (α-SMA) and collagen. Then, SAHA was used to interfere with TGF-β-treated and non TGF-β-treated cells and it was found that SAHA significantly reduced the protein expressions of α-SMA, collagen I, and fibronectin in fibroblasts (Figure 2A). Besides, the RNA levels of α-SMA, collagen I and fibronectin in fibroblasts were consistently decreased following SAHA administration (Figure 2B). Therefore, it was demonstrated that SAHA reduced myofibroblasts formation and inhibited extracellular matrix (ECM) deposition by regulating fibroblasts transdifferentiation.

SAHA is Pro-Apoptotic in Human Fibroblast Through Enhancing Caspase-3

Excessive proliferation of fibroblasts also leads to the aggravation of scar hyperplasia and the obstruction of tissue repair, and the effective regulation of the amounts of apoptotic fibroblasts is conducive to the amelioration of tissue fibrosis. To explore whether SAHA mediates fibroblast apoptosis, the impact of SAHA with different concentrations on fibroblast viability was detected using CCK-8 assay. The results showed that the viability of fibroblasts was inhibited *via* concentration dependent SAHA (Figure 3A). Then, flow cytometry was used to quantify apoptotic cells treated with SAHA and TGF-β and it was found that TGF-β improved the level of apoptosis, while SAHA increased the number of apoptotic fibroblasts (Figure 3B). The regulated mechanism of SAHA on apoptosis was further explored, and apoptosis relative factors in fibroblasts treated

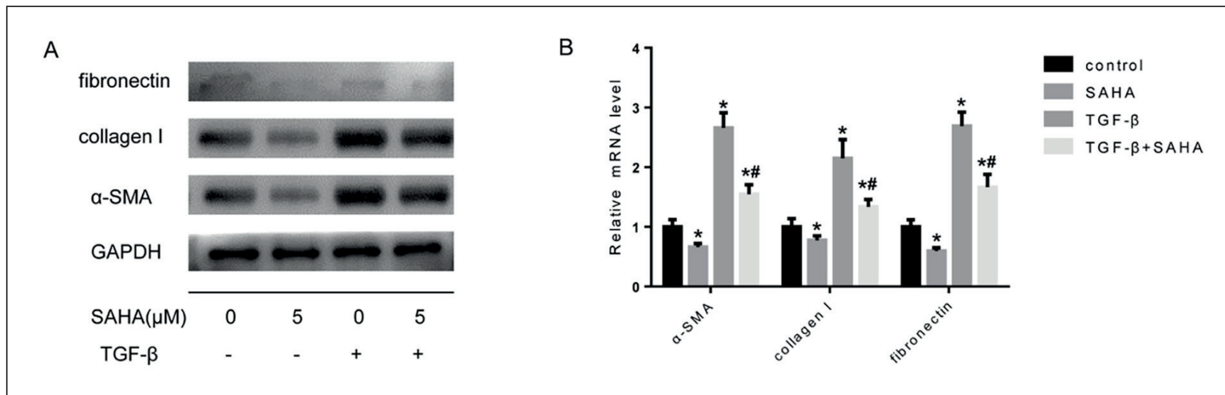

Figure 2. SAHA inhibits TGF-β-induced myofibroblast transdifferentiation. **A,** Representative Western blotting of collagen I, fibronectin and α-SMA in the control, SAHA, TGF-β and TGF-β+SAHA group. **B,** Representative mRNAs of collagen I, fibronectin and α-SMA in the control, SAHA, TGF-β and TGF-β+SAHA group. "*" means *vs.* control group with statistical significance. "#" means *vs.* TGF-β group with statistical significance.

with SAHA and TGF-β were measured. The results showed that the protein and RNA levels of bax-2 and caspase-3 significantly increased, and bcl-2 decreased after SAHA treatment (Figure 3C and 3D). Thus, SAHA mediates fibroblast apoptosis by enhancing the role of Caspase-3.

SAHA Attenuates Epidural Fibrosis in Rat Model Following Laminectomy

The anti-fibrotic role of SAHA was investigated using the rat laminectomy model *in vivo* and the fibrosis level of epidural scar tissue protein was measured in the SAHA group and

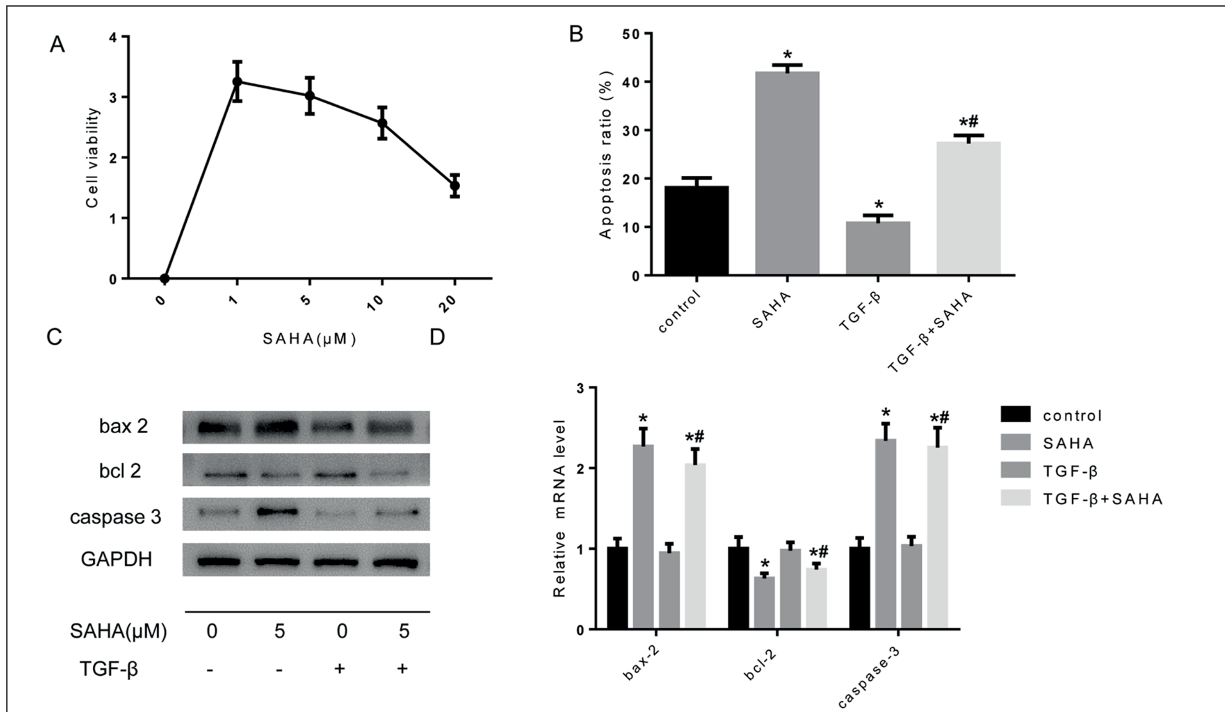

Figure 3. SAHA is pro-apoptotic in human fibroblast through enhancing Caspase-3. **A,** Cell viability alteration in 1 μM, 5 μM, 10 μM and 20 μM SAHA, respectively. **B,** Cell apoptosis ratio in the control, SAHA, TGF-β and TGF-β+SAHA group. **C,** Representative Western blotting of caspase-3, bax 2 and bcl 2 in the control, SAHA, TGF-β and TGF-β+SAHA group. **D,** Representative RNA levels of caspase-3, bax 2 and bcl 2 in the control, SAHA, TGF-β and TGF-β+SAHA group. "*" means *vs.* control group with statistical significance. "#" means *vs.* TGF-β group with statistical significance.

the control group at 7 days post operation. The results exhibited that the protein levels of α-SMA, collagen I, and fibronectin in the SAHA group were significantly lower than those in the control group 7 days following surgery (Figure 4A and 4B). Besides, the apoptosis proteins were detected at 3 days post operation, finding that bax-2 and caspase-3 significantly elevated but bcl-2 remarkably reduced (Figure 4C and 4D). Therefore, it was demonstrated that SAHA exerts the effect of inhibiting fibrosis by promoting apoptosis and myofibroblast activation after laminectomy in rats.

Discussion

Fibrosis hyperplasia usually occurs in wound repair but accumulating fiber activation triggers a great deal of collagen generation, resulting in abundant scar tissue and impacting healing. Fibroblast takes a critical role in the secretion of ECM and the regulation of the stability of fibrosis scar. Epidural fibrosis is accompanied with alterations in fibroblasts, including activation and differentiation of fibroblasts, proliferation and apoptosis, as well as accumulation of ECM. Severe epidural fibrosis leads to compression of the spinal cord and nerve root tissue, resulting in severe neurological dysfunction and disorder. For the complicated mechanism of epidural fibrosis, ECM increase and cell proliferation are widely accepted as the specific features during its process.

HDACs are involved in many physiological processes[18,19]. HDACs dysfunction is linked to a variety of diseases, including cancer, cardiac hypertrophy[20], and diabetes[21]. HDACIs are molecules that bind with HDACs to interfere or block its function. HDACIs regulate gene expression and apoptosis through acetylation of histone. SAHA, as a HDACI, is effective in the treatment

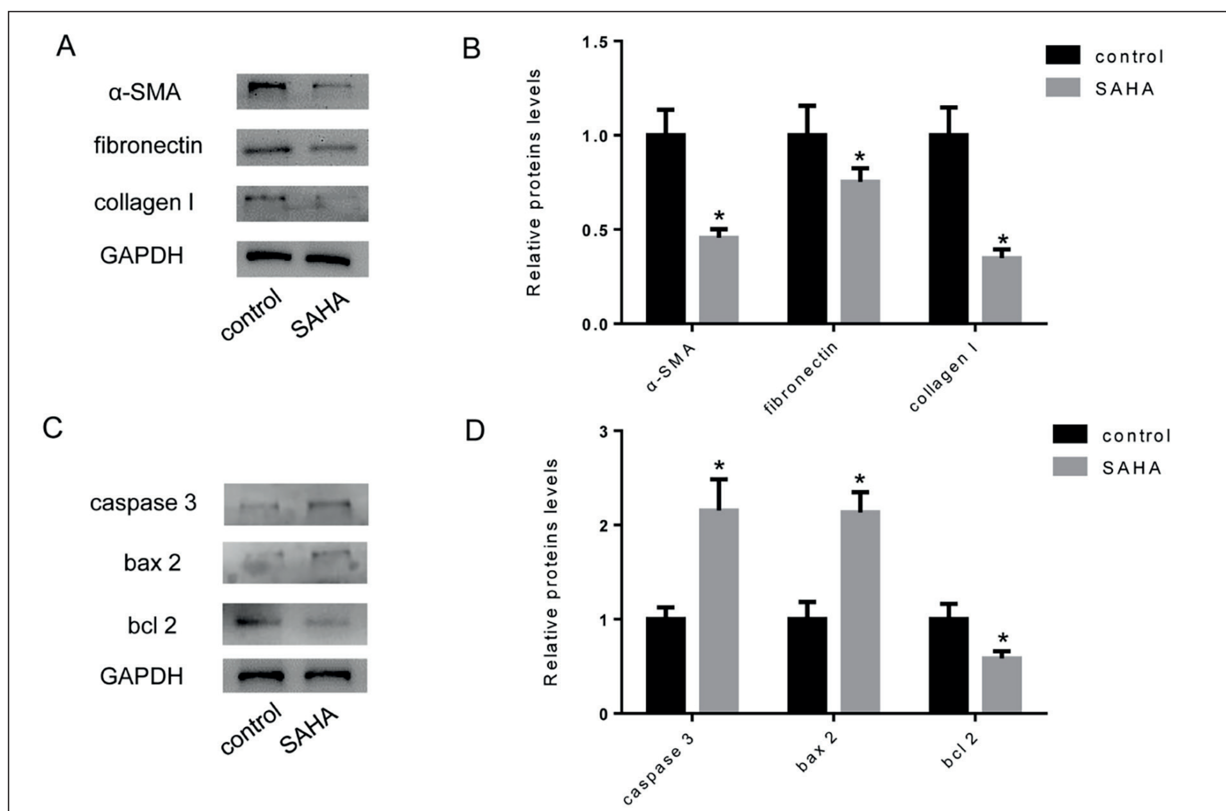

Figure 4. SAHA attenuates epidural fibrosis in rat model following laminectomy. **A,** Representative Western blotting of collagen I, fibronectin and α-SMA in the control and SAHA group at 7 days following surgery. **B,** Representative protein analysis of collagen I, fibronectin and α-SMA in the control and SAHA group at 7 days following surgery. **C,** Representative western blotting of bax 2, caspase 3, and bcl 2 in the control and SAHA group at one week following surgery. **D,** Representative protein analysis of bax 2, caspase 3, and bcl 2 in the control and SAHA group at 7 days following surgery. "*" means *vs.* control group with statistical significance.

of pulmonary fibrosis. SAHA inhibited myofibroblast differentiation in primary human lung fibroblasts[22], downregulated collagen III expression, increased lung fibroblast apoptosis in bleomycin-induced pulmonary fibrosis mice[23], and upregulated cyclooxygenase-2 and prostaglandin E2 expression to inhibit pulmonary fibrosis[24]. So, it is hypothesized that SAHA may decrease pro-fibrotic signaling loops and promote the effect of fibroblast apoptosis.

From the result of the different levels of acetylated histone 3 and acetylated α-tubulin in both AHF and HEF, it is evident that cells treated with SAHA have higher acetylated histone 3 and acetylated α-tubulin expression; and acetylated histone 3 and acetylated α-tubulin in cells treated with TGF-β were at low level. Regarding the fibroblast culture, TGF-β was widely used to mimic the pathophysiology of fibroblast activation *in vitro*. SAHA treatment to fibroblast is used to explore the influence of myofibroblast transdifferentiation *via* TGF-β induction. The result indicated that SAHA had an ability to interfere the activation of myofibroblast with collagen I, fibronectin, and α-SMA decrease. Promoting fibroblast apoptosis can inhibit fibroplasia, so SAHA of different concentrations was utilized to test several targets corresponding to the cell apoptosis. It was exhibited that the concentration-dependent SAHA reduced fibroblast viability and the apoptosis fibroblast increased with SAHA treatment compared with the control one and TGF-β treatment. As anticipated, the pro-apoptosis effect of SAHA to the epidural fibrosis depended on overexpression of caspase-3 and inhibition of bcl-2. Recent studies have suggested that SAHA increased lung fibroblast apoptosis in idiopathic pulmonary fibrosis. This study confirmed again that SAHA exerted pro-apoptosis function and could reduce TGF-β-induced fibrosis process.

Moreover, to verify SAHA therapeutic effect of epidural fibrosis *in vivo*, SAHA was used to treat rats following laminectomy. At 3 days post laminectomy, the result demonstrated that SAHA could influence apoptosis relative protein expression. To explore whether SAHA both have the capacity to decrease myofibroblasts activation and ECM deposition, ECM expression was analyzed in epidural scar including α-SMA, collagen I, and fibronectin. Previous studies have shown that α-SMA, collagen I and fibronectin can participate in fibrosis induced by TGF-β regulating. Our findings also lead to strong support to the view that ECM is reduced by the decreased expression of HDAC. Hence, the novelty of the present study was that we utilized AHF and HEF to explore acetylated histone 3 and acetylated α-tubulin alterations caused by HDAC3 activity and speculated that HDAC3 inhibition might modulate fibroblasts viability and transdifferentiation. Moreover, SAHA was firstly confirmed to exert anti-fibrosis in epidural fibrosis model by reducing TGF-β-induced ECM accumulation and accelerating fibroblasts apoptosis both *in vivo* and *vitro*, whereas there are few studies referring to HDACIs function in epidural fibrosis. Hopefully, SAHA is a promising inhibitor that suppresses the influence of ECM to alleviate the fibroplasia in epidural fibrosis.

To summary, it would be gracefully to seek a drug that works by inhibiting ECM accumulation and elevating cell apoptosis *via* inhibition of HDAC to attenuate epidural fibrosis. The role of SAHA treatment on the fibrosis model both *in vivo* and *in vitro* was systematically evaluated. Together, these results revealed a knowledge facilitating investigations that SAHA may be a useful method to treat the epidural fibrosis.

Conclusions

These results indicated that SAHA inhibited HDAC 3 expression, decreased TGF-β effect, and enhanced caspase-3 in fibroblasts, leading reduction of myofibroblast activation and apoptosis elevation. Hence, SAHA ameliorated epidural fibrosis development.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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