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Original Paper

High Concentration of Aspirin Induces Apoptosis in Rat Tendon Stem Cells via Inhibition of the Wnt/β-Catenin Pathway

Yunjiao Wang[a] Hong Tang[a] Gang He[a] Youxing Shi[a] Xia Kang[a] Jingtong Lyu[a] Mei Zhou[a] Min Zhu[a] Jiqiang Zhang[b] Kanglai Tang[a]

[a]Department of Orthopeadics, Southwest Hospital, Third Military Medical University, Chongqing, [b]Department of Neurology, Third Military Medical University, Chongqing, China

Key Words
Tendon stem cells • Aspirin • Wnt/β-catenin pathway • Apoptosis

Abstract
Background/Aims: Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in clinical practice to relieve fever and pain. Aspirin, as a representative NSAID, has been widely used in the treatment of tendinopathy. Some reports have demonstrated that aspirin can induce apoptosis in cancer cells. However, evidence regarding aspirin treatment for tendinopathy, especially the effect of this treatment on tendon stem cells (TSCs), is lacking. Understanding the effect of aspirin on tendinopathy may provide a basis for the rational use of NSAIDs in clinical practice. The aim of our study was to determine whether aspirin induces apoptosis in rat TSCs via the Wnt/β-catenin pathway. ***Methods:*** First, we used flow cytometry and fluorescence to detect TSC apoptosis. Protein expression of the apoptosis-related caspase-3 pathway was investigated via western blot analysis. Next, we used western blotting to determine the effect of aspirin on the Wnt/β-catenin pathway. We used immunostaining to detect the levels of Bcl2, cleaved caspase-3, and P-β-catenin in the Achilles tendon. Finally, we used flow cytometry, fluorescence, and western blotting to investigate the aspirin-induced apoptosis of TSCs via the Wnt/β-catenin pathway. ***Results:*** Aspirin induced morphological apoptosis in rat TSCs via the mitochondrial/caspase-3 pathway and induced cellular apoptosis in the Achilles tendon. Apoptosis was partly reversed after adding the Wnt signaling activator Wnt3a and lithium chloride (LiCl, a GSK-3β inhibitor). Aspirin administration led to a dose-dependent increase in COX-2 expression. Apoptosis was promoted after adding the COX-2 inhibitor NS398. ***Conclusion:*** The Wnt/β-catenin pathway plays a vital role in aspirin-induced apoptosis by regulating mitochondrial/caspase-3 function. Elevating COX-2 levels may protect cells against apoptosis. More importantly, the results remind us to consider the apoptotic effect of aspirin on TSCs and tendon cells when aspirin is administered to treat tendinopathy. The relationship between the positive and negative effects of aspirin remains a subject for future study.

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Kanglai Tang

Department of Orthopedic Surgery, Southwest Hospital, Third Military Medical University
Gaotanyan Street. 30, Shapingba District, Chongqing 400038 (China)
E-Mail tangkanglai@hotmail.com

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Introduction

Aspirin, which for over 150 years has been one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs) [1, 2], has been reported to display a wide range of effects, such as antipyretic and analgesic effects in the cardiovascular system [3] and central nervous system, as well as apoptotic effects in some cancers, such as breast cancer [4, 5]. NSAIDs inhibit function in the cyclooxygenase enzymes (COX-1 and/or COX-2), which catalyze prostaglandin formation, thereby reducing inflammation and pain. NSAIDs are therefore effective at relieving symptoms in conditions such as rheumatoid arthritis.

Tendons are fibrous connective tissue bands that attach muscle to bone and convey mechanical force, permitting joint and whole-body motion. As the result of constant mechanical loading, tendons can often become affected by various pathologies, collectively called tendinopathy [6-8]. Several mechanisms have been identified in the pathogenesis of tendinopathy [9]. Sudden increase in loading forces, long-distance running, tendon overuse [10], inherited genes or gene variants [11], metabolic disorders [12], hormone imbalance, and loss of control over cellular homeostasis [13] can all lead to tendinopathy. Tendinopathy is one of the most common and most challenging problems for surgeons in orthopedics and sports medicine. Studies have reported that tendinopathy accounts for approximately 30% of sports injuries, and its pathogenesis is highly related to inflammation and degradation of connective tissue [14, 15].

Traditionally, tendons were thought to contain only tenocytes, but current theories positing that the maintenance and repair of adult tissues is reliant on small populations of resident stem cells have become widely accepted [16, 17]. Tendon stem cells (TSCs) or progenitor cells were first reported in human and mouse tendons in 2007, and were then subsequently confirmed in rat and rabbit tendons [18-20]. TSCs differ from tenocytes in terms of their colony-forming ability, self-renewal ability, and multidifferentiation potential, which enables them to differentiate into tenocytes, adipocytes, chondrocytes, and osteocytes [21]. Therefore, the viability and tenogenic differentiation of TSCs are closely associated with the maintenance of the tendon microenvironment and the development of tendinopathy.

Although NSAIDs are commonly used in clinical practice to reduce inflammation and alleviate musculoskeletal pain, little evidence actually supports this treatment. Similarly, little is known regarding the effects of NSAIDs on TSCs and tendinopathic tendons. Some reports have revealed that aspirin can induce apoptosis in cancer cells, and in our previous experiments, we found that aspirin can also induce apoptosis in TSCs. This effect is contrary to aspirin's anti-inflammation and pain relief properties, as in general, TSC apoptosis interferes with healing in tendinopathy. To evaluate the influence of aspirin on TSC apoptosis, we first determined whether aspirin could indeed induce TSC apoptosis. Second, we analyzed the role of the Wnt/β-catenin pathway in aspirin-induced apoptosis. Lastly, we analyzed the effects of aspirin on COX-2 and the role of COX-2 in apoptosis.

Materials and Methods

Ethics statement

Eight-week-old Sprague-Dawley rats weighing 200–250 g were used. The rats were housed under a 12-h light/dark cycle in a pathogen-free area with free access to water and food. All animals were treated according to institutional guidelines for laboratory animal treatment and care. All experimental procedures were approved by the Animal Research Ethics Committee of the Third Military Medical University, chongqing, China.

Animal model

A total of 24 male Sprague-Dawley rats were used. Aspirin (30 mg/d) was administered to each rat via gavage. At weeks 2 and 4 after aspirin administration, 8 rats from each group were killed and Achilles tendons were harvested for immunostaining.

Isolation and culture of rat TSCs

A total of 12 male Sprague-Dawley rats were used for isolation of rat TSCs *in vitro*. Isolation of rat TSCs was performed as previously described [6, 14]. Briefly, intact flexor digitorum longus tendons from both hind feet were dissected after euthanasia. Only the mid-substance tissue was collected, and the peritendinous connective tissue was carefully removed. The mid-substance tissue was minced in sterile phosphate-buffered saline (PBS) and digested in 3 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO) for 2.5 h at 37°C. A 70-mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) was used to yield a single-cell suspension. The released cells were washed in PBS and centrifuged at 300 × *g* for 5 min before being resuspended in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA). Increasing dilutions of the isolated cells were plated, grown for 2 days at 37°C in 5% CO_2, and then washed twice in PBS to remove nonadherent cells. On day 7 of culture, the cells were trypsinized with trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA), mixed together, and cultured as passage 0 cells. Cells from passage 3 (P3) were used in subsequent experiments. TSCs were seeded onto 6-well plates for cell staining and flow cytometric analysis and onto 10-cm-diameter petri dishes for protein extraction. Culture medium with or without aspirin (Sigma-Aldrich) dissolved in dimethyl sulfoxide was changed every 3 days throughout the experiments.

TSCs were seeded in a 6-well plate at a density of 6×10^4 cells/well for each experiment, and the cells were cultured in the above-described medium with 0, 0.25, 0.5, 1, 2, or 5 mM aspirin for 24 h or with 5 mM aspirin for 0, 1, 2, 4, 8, 12, 16, 20, or 24 h. In the inhibition studies, TSCs were preincubated with human recombinant Wnt3a protein (0.1 µg/ml) and GSK-3β inhibitor or LiCl (0.5 mM) or the COX-2 inhibitor NS-398 (5 µM) for 1 h before the addition of aspirin (5 mM) for 24 h.

Morphological assessment of TSC apoptosis

TSCs treated with various agents for nuclear condensation and fragmentation were studied by staining with Hoechst 33342 as previously described [22]. Briefly, TSCs were washed by PBS and dye buffers were added to cells. Cells were then stained with Hoechst 33342 in the dark at 4°C for 10 min, and morphological changes were observed using fluorescence microscopy (Olympus IX70 Microscope, Olympus Corp., Tokyo, Japan; RS Image Express processing software, version 4.5). Apoptotic cells were defined as those cells having condensed, fragmented nuclei and undergoing cell shrinkage, shown in bright blue.

Flow cytometric analysis of TSC apoptosis

Phosphatidylserine exposure on the surface of plasma membranes was detected using an Annexin V-FITC Apoptosis Detection Kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocols. Briefly, the cells were harvested with pancreatin free of EDTA, washed twice with ice-cold PBS, and resuspended in 100 µl of binding buffer. The cells were then incubated with 5 µl of Annexin V-FITC solution and 5 µl of propidium iodide (PI) staining solution at room temperature for 10 min in the dark. Finally, another 400 µl of binding buffer was added, and the cells were immediately analyzed by bivariate flow cytometry using a FACScan-LSR flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA). At least 1×10^4 cells per sample were acquired and analyzed.

Protein extraction and western blotting

The cells were washed twice with PBS and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 150 mM NaCl) containing a mixture of proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL). Total protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc.), and equal amounts of proteins (30 µg/lane) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. Membranes were then blocked by incubation with 5% nonfat milk containing 0.1% tris-buffered saline with Tween 20 (TBST)

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for 2 h at room temperature. The membranes were then incubated sequentially with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-Bcl2 (Proteintech, Rosemont, IL, USA), anti-BAX (Proteintech, Rosemont, IL, USA), anti-Cleavage-Caspase-3 (Cell Signaling, Danvers, MA), anti-GSK-3B (Proteintech, Rosemont, IL, USA), anti-phospho-GSK-3B (ser9) (Bioss Antibodies, Woburn, MA), anti-β-catenin (Proteintech, Rosemont, IL, USA), anti-phospho-β-catenin (ser33/37) (Bioss Antibodies, Woburn, MA), anti-Cyclin D1 (Proteintech, Rosemont, IL, USA), anti-c-myc (Proteintech, Rosemont, IL, USA), and anti-COX-2 (Proteintech, Rosemont, IL, USA). β-actin (Proteintech, Rosemont, IL, USA) was used as an internal control. Following primary antibody incubation, membranes were washed 3 times in 0.1% TBST and incubated in goat anti-rabbit IgG (H&L)-horseradish peroxidase conjugate (1:2000) for 2 h at room temperature. The proteins were visualized, and images were captured using a LI-COR Odyssey Imager (LI-COR Biosciences, Lincoln, NE).

Immunostaining

Immunostaining analysis was performed by immunofluorescent tagging for Bcl2, cleaved caspase-3 and phospho-β-catenin in the tissue sections following our existing protocol. Briefly, the frozen sections were fixed with ice-cold acetone. Sections were then washed 3 times in PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with 5% bovine serum albumin in PBS with Tween 20. Immunostaining was performed with rabbit anti-rat caspase-3 (1:100), rabbit anti-rat Bcl2 (1:100), and rabbit anti-rat phospho-β-catenin (1:100) overnight at 4°C. The sections were washed 3 times in PBS and incubated for 1 h at room temperature in the dark with Cy3-conjugated goat anti-rabbit IgG (H&L) (Abcam, Cambridge, UK) in PBS, and the cell nuclei were counterstained with 0.5 μg/ml DAPI (Beyotime, Shanghai, China) for 2 min. The sections were covered with mounting medium, and the coverslips were sealed with nail polish to prevent drying and fluorescence quenching. The samples were observed and photographed under a fluorescence microscope (Leica, Wetzla, Germany).

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Student's t-test was used to compare means between two groups. Multiple comparisons were made using one-way analysis of variance followed by Fisher's test. A p-value < 0.05 was considered statistically significant.

Results

Aspirin induces apoptosis in TSCs

Fluorescence microscopy and flow cytometry were used after cell staining. Hoechst 33342 staining showed that most cells in the control group had normal and regular nuclei, while in the treatment groups, especially in the groups treated with 1, 2, and 5 mM aspirin, more cells had condensed and lightened chromatin fragments compared with the control group ($p < 0.05$). These findings were further confirmed by flow cytometry. Additional early apoptotic nuclei staining with Annexin V occurred with increasing concentrations of aspirin, especially with 1, 2, and 5 mM aspirin. Both Hoechst 33342 staining and Annexin V-FITC staining suggested an apoptotic effect of aspirin on TSCs (Fig. 1A, B).

Aspirin-induced apoptosis via the mitochondrial/caspase-3 pathway

The mitochondrial/caspase-3 pathway is one of the main pathways related to apoptosis. Thus, we first investigated whether aspirin-induced apoptosis occurs via this pathway. We observed the expression of Bcl2, BAX, and caspase-3, which are key proteins of the mitochondrial/caspase-3 pathway, as our target proteins, using western blotting and immunofluorescence. Western blotting showed that anti-apoptosis protein Bcl2 decreased suddenly when the concentration of aspirin was 2 and 5 mM. However, there was no increase in the pro-apoptosis protein BAX, which is downstream of and inhibited by Bcl2.

Unexpectedly, the results showed that aspirin treatment for 16, 20, and 24 h increased the expression of BAX (Fig. 2B). Additionally, we observed the expression of cleaved caspase-3, the activated form of pro-caspase-3, the release of which is regulated by Bcl2 and

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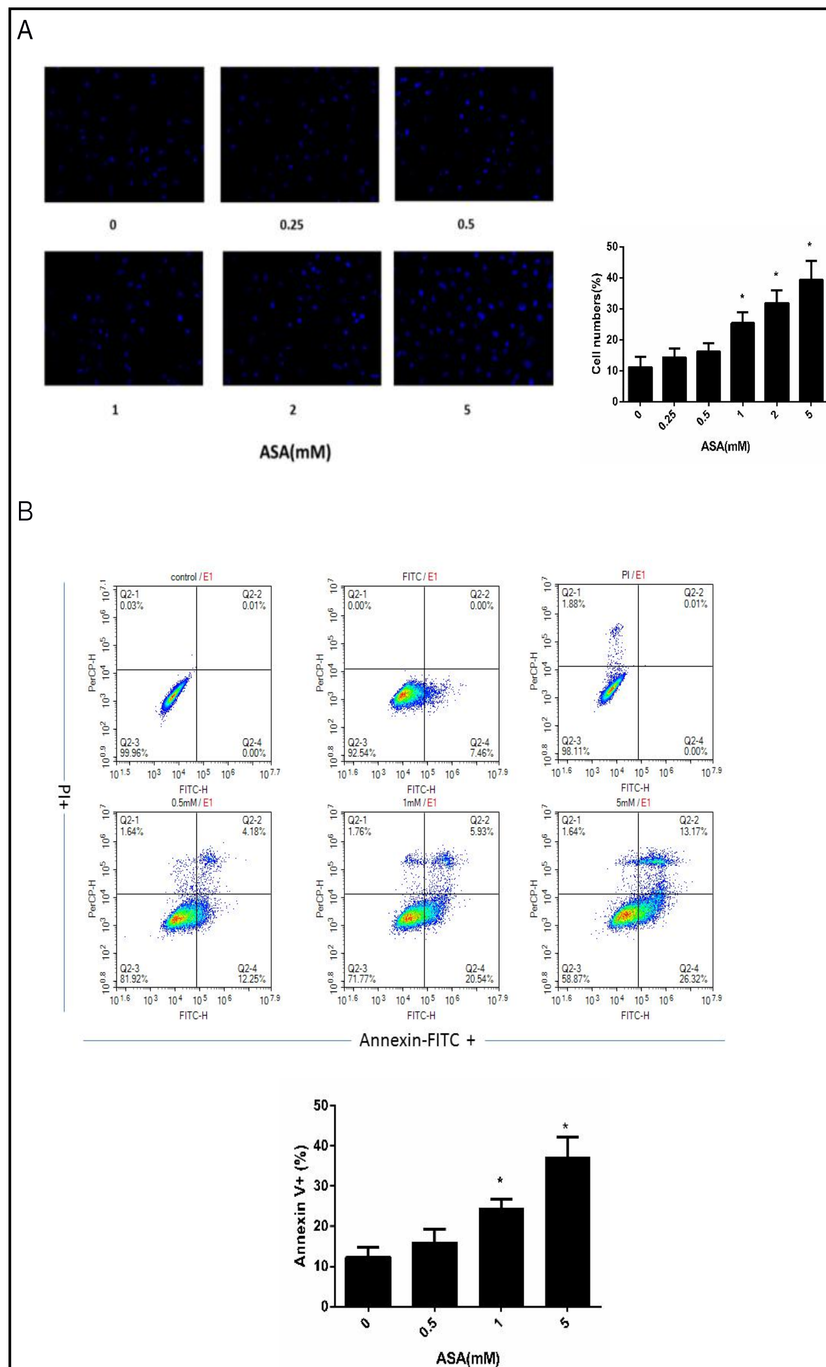

Fig. 1. Effects of different concentrations of aspirin on TSC apoptosis. Hoechst 33342 staining and Annexin V-FITC staining show that high concentrations of aspirin had apoptotic effects on TSCs. (A, B) TSCs treated with 1, 2, or 5 mM aspirin showed more condensed and lightened nuclear fragments, but no similar trends appeared in cells treated with 0.25 or 1 mM aspirin. (C, D) Annexin V-FITC staining detected by flow cytometry showed that more early and late apoptotic cells appeared in the 1, 2, and 5 mM aspirin groups than in the low aspirin concentration groups. Cells stained with FITC+/PI- represent the early apoptotic cells, and those stained with FITC+/PI+ represented the late apoptotic cells. The data are presented as the mean ± SD of three independent experiments. *: $p<0.05$, n = 3.

BAX proteins. The results showed that the level of caspase-3 increased in a dose-dependent and time-dependent manner (Fig. 2A, B). These western blotting observations are consistent with the results obtained by immunofluorescence, which showed decreased expression of Bcl2 and increased expression of cleaved caspase-3 in rat tendons 4 weeks after aspirin intake compared with expression after two weeks or after no administration of aspirin. The western blotting and immunofluorescence results showed that aspirin induced the apoptosis of TSCs and tendon cells through the mitochondrial/caspase-3 pathway.

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Aspirin inhibited the canonical Wnt/β-catenin pathway of TSCs and tendon cells

Previous studies have reported that the Wnt/β-catenin pathway is related to cell development and proliferation [23-25]. Therefore, the present study considered the Wnt/β-catenin pathway as a target pathway for aspirin-induced apoptosis. We observed changes in the levels of Dickkopf-related protein 1 (DKK1), P-GSK-3β (ser9), P-β-catenin (ser33/37), and the downstream nuclear proteins of C-myc and Cyclin D1 through western blotting and immunofluorescence. From the results, we found that the expression of P-GSK-3β, C-myc, and cyclin D1 decreased in a time- and dose-dependent manner when TSCs were treated with high concentrations of aspirin (1, 2, or 5 mM) or 5 mM aspirin for 16, 20, or 24 h.

At the same time, levels of DKK1 and P-β-catenin showed an inverse trend compared to P-GSK-3β when TSCs were treated with 5 mM aspirin for 16, 20, or 24 h, when the immunofluorescence assays showed significantly increased expression of P-β-catenin in rat tendons 4 weeks after aspirin intake compared with levels in the non-aspirin group and the 2-week group. The above results were in accordance with the changes in the levels of proteins in the mitochondrial/caspase-3 pathway, and indicated that aspirin inhibits the canonical Wnt/β-catenin pathway (Fig. 3).

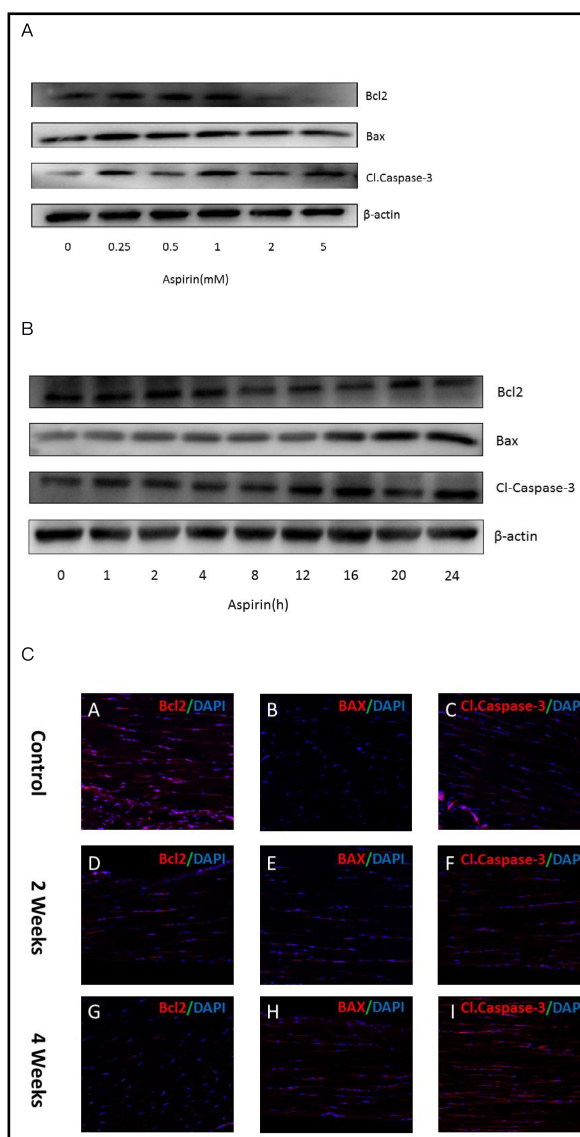

Fig. 2. Aspirin activated the mitochondrial/caspase-3 pathway in TSCs and tendon cells. (A, B) Western blotting showed that a decrease in Bcl2 and an increase in caspase-3 occurred when TSCs were treated with high concentrations of aspirin (1, 2 or 5 mM). Levels of BAX and caspase-3 increased in TSCs treated with 5 mM aspirin for 16, 20 or 24 h. (C) Representative images of immunofluorescence staining of Bcl2 and caspase-3 in the Achilles tendon with 30 mg/d aspirin administration for 2 weeks and 4 weeks. The data are presented as the mean ± SD of three independent experiments. *: $p<0.05$ vs. control, #: $p<0.05$ vs. aspirin, n = 3.

Activation of the canonical Wnt/β-catenin pathway attenuated aspirin-induced apoptosis

To investigate if the Wnt/β-catenin pathway mediates aspirin-induced apoptosis, we added two activators of the Wnt/β-catenin pathway—wnt3a and LiCl—to TSCs before aspirin treatment. First, we ensured the activation effect of wnt3a and LiCl on the pathway. The results showed that Wnt3a and LiCl elevated the levels of P-GSK-3β (ser9), C-myc, and Cyclin D1, while lowering the level of P-β-catenin (ser33/ser37). In addition, we explored whether the apoptotic effect was attenuated after these activators were added. As expected, apoptosis in the group with activators was lower than that in the aspirin-only group. The two activators attenuated the aspirin-induced

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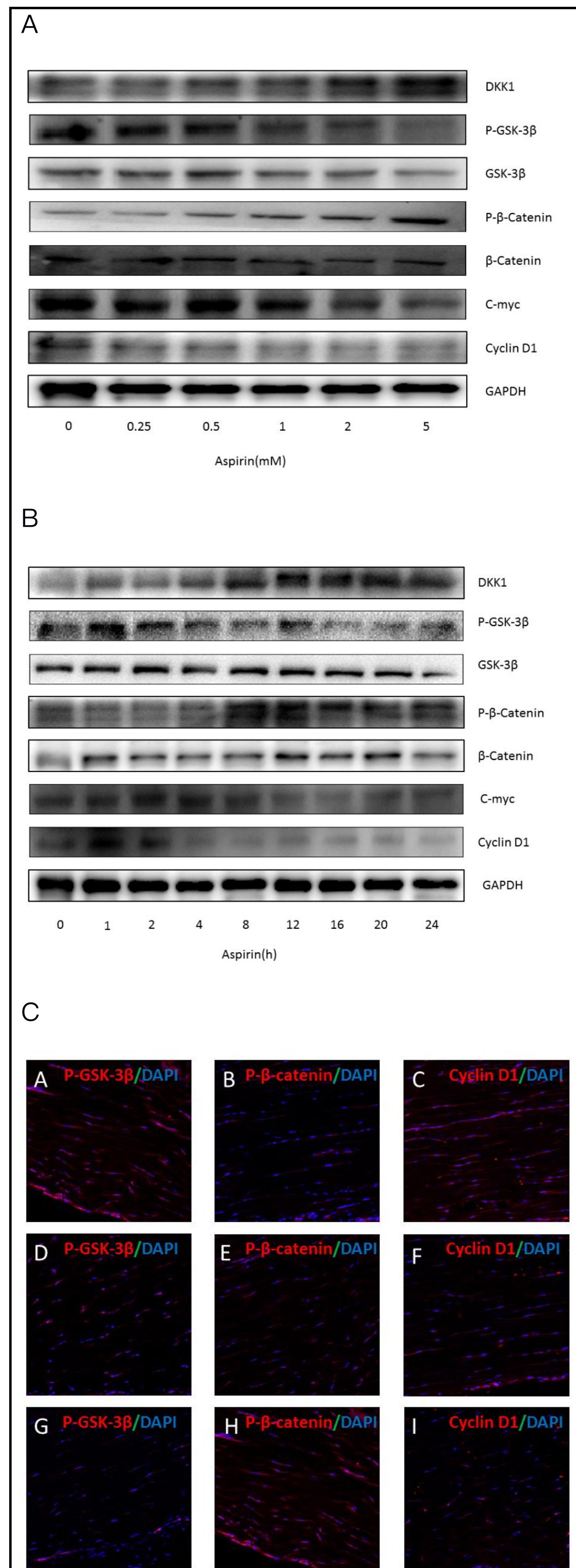

Fig. 3. Aspirin inhibited the canonical Wnt/β-catenin pathway in rat TSCs. (A, B) Western blotting showed that P-GSK-3β, Cyclin D1, and C-myc decreased and that P-β-catenin increased when TSCs were treated with high concentrations of aspirin (1, 2, or 5 mM). C-myc and P-β-catenin increased in TSCs treated with 5 mM aspirin for 16, 20, or 24 h. (C) Representative images of immunofluorescence staining of P-β-catenin in the Achilles tendon with 30 mg/d aspirin administration for 2 weeks. The data are presented as the mean ± SD of three independent experiments. *: $p<0.05$ vs. control, #: $p<0.05$ vs. aspirin, n = 3.

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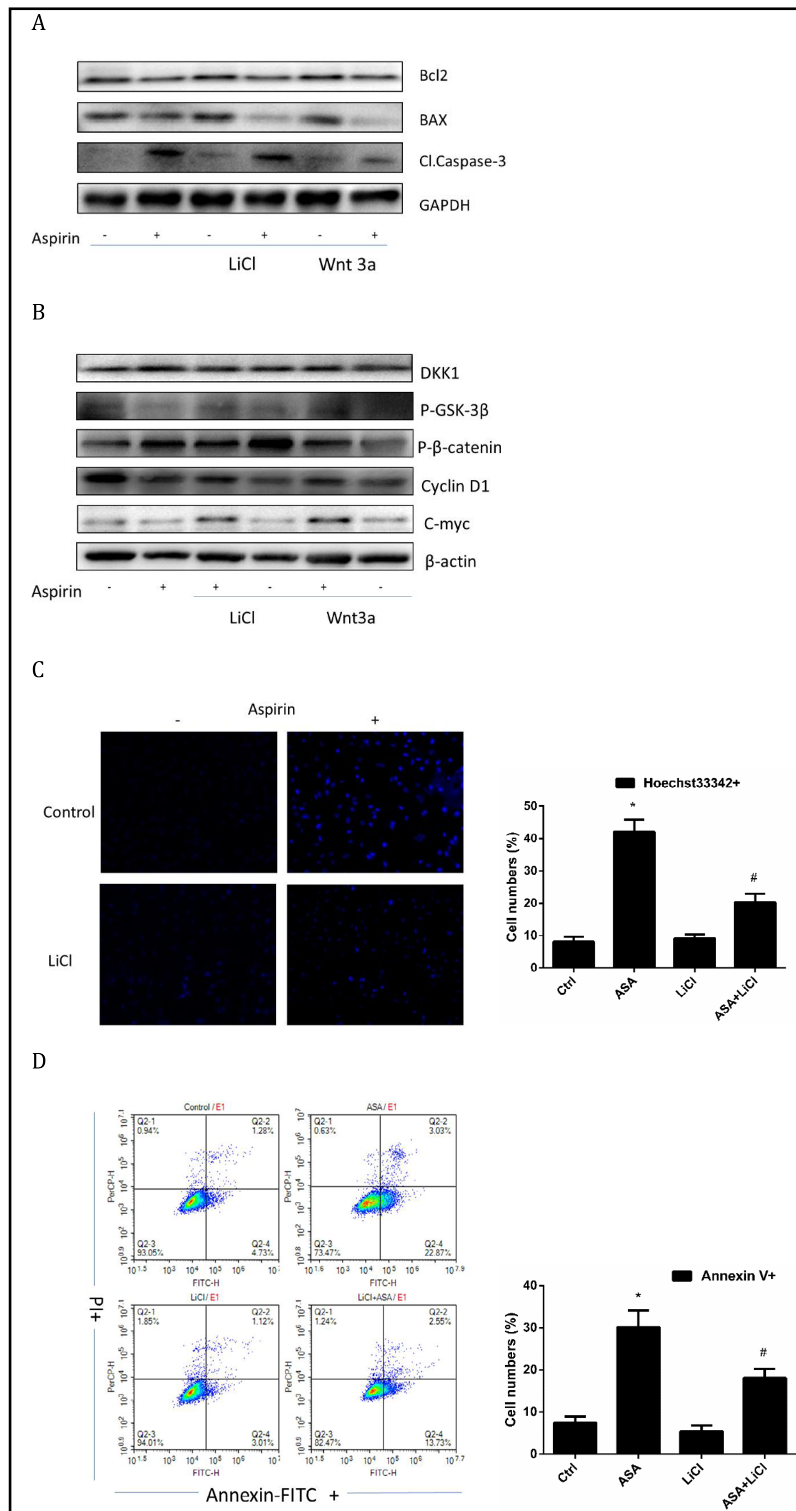

Fig. 4. Wnt3a and LiCl attenuated the aspirin-induced inhibition of Wnt/β-catenin signaling and apoptosis of TSCs. [A] Wnt3a and LiCl elevated P-GSK-3β (ser9), Cyclin D1, and C-myc, and they decreased P-β-catenin (ser33/ser37). (B-D) Wnt3a and LiCl also antagonized the aspirin-induced apoptosis of TSCs. (B) Changes in Bcl2, BAX, and caspase-3. (C, D) Hoechst 33342 staining (C) and flow cytometric analysis (D) of Annexin V-FITC staining. The data are presented as the mean ± SD of three independent experiments. *: $p<0.05$ vs. control, #: $p<0.05$ vs. aspirin, n = 3.

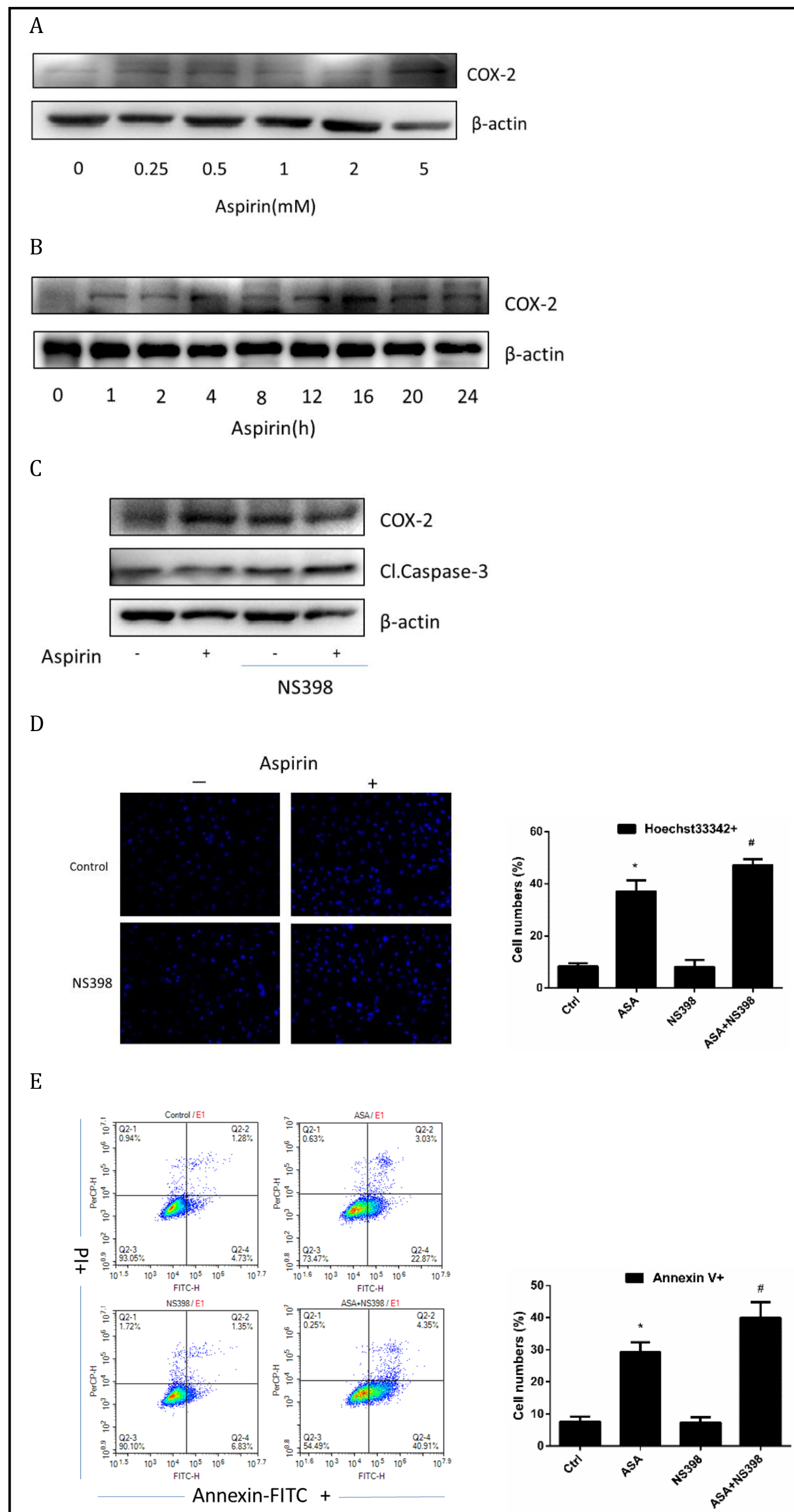

Fig. 5. Elevated levels of COX-2 induced by aspirin antagonized apoptosis induced by aspirin. (A, B) Aspirin elevated the level of COX-2 in a dose-dependent (A) and time-dependent (B) manner. (C-E) Western blotting (C), cell staining (D), and flow cytometric analysis (E) showed that treatment with the COX-2 inhibitor NS398 promoted TSC apoptosis compared with treatment with only aspirin. The data are presented as the mean ± SD of three independent experiments. *: $p<0.05$ vs. control, #: $p<0.05$ vs. aspirin, n = 3.

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downregulation of Bcl2 and cleaved caspase-3 and the upregulation of BAX. These results were also confirmed by Hoechst 33342 and Annexin V/FITC staining, and the number of apoptotic cells decreased. Collectively, these results suggested that aspirin-induced apoptosis is mediated by DKK1/Wnt/β-Catenin (Fig. 4).

Aspirin promoted COX-2 expression, and COX-2 weakened aspirin-induced apoptosis

It is known that aspirin can non-selectively inhibit COX-2 activity via inhibition of PEG2. In our preexperiment, we unexpectedly found that the expression of COX-2 dose-dependently increased but time-dependently decreased. We believe that the results may be affected by the non-inflammation environment and early cell stress response.

To investigate the role of increased expression of COX-2 in aspirin-induced TSC apoptosis, we added the COX-2 inhibitor NS398 prior to aspirin treatment. We found that the apoptotic rate in the NS398 treatment group clearly increased compared to that of the aspirin-only group, which was confirmed by Hoechst 33342, Annexin V/FITC staining, and mitochondria-related protein expression. We suppose that elevated levels of COX-2 were stimulated by aspirin-induced apoptosis and that those increased levels weakened the aspiring-induced apoptotic effect (Fig. 5).

Discussion

NSAIDs have been widely used for the clinical treatment of the musculoskeletal system, but there is little known about their effect on tendon healing [26]. Aspirin is a classic, representative NSAID, and so understanding its effects on TSCs can give us useful information regarding other NSAIDs. The cellular niche of tenocytes and TSCs is significant to tendinopathy; for example, Del Buono et al. reported that increasing metalloproteases may induce tendinopathy [27] and Lavagnino et al. showed that a hypoxic environment may lead to inability to respond to strain-based rehabilitation modalities in chronic tendinopathy [28]. TSCs are at the core of the cause, development, and healing of tendinopathy. Aspirin may hinder the healing of tendinopathy via inhibition of cell proliferation [29], induction of apoptosis, inhibition of the migration of tendon cells [30], or inhibition of the synthesis and secretion of type I collagen and other important tendon matrix proteins. Thus, any factors that affect the proliferation, apoptosis, or differentiation of TSCs and their cellular niche can indirectly alter the prognosis of tendinopathy. Chen et al. revealed that aspirin can induce apoptosis in cancer cells [31], but there have been no reports about the effects of aspirin on TSCs. Therefore, whether aspirin has anti-inflammatory and analgesic effects, as well as a simultaneous effect on apoptosis, is a key scientific issue that may have implications for tendinopathy therapy and may caution orthopedists and doctors in sports medicine regarding the use of aspirin.

To determine the effect of aspirin on TSCs, we first investigated the Hoechst 33342 staining and Annexin V/FITC staining of aspirin-treated TSCs *in vitro*. The apoptotic cells stained with Hoechst 33342 showed condensed DNA and fragmented, lighter nuclei. Annexin V is a phospholipid-binding protein that can selectively bind phospholipids on the outer membrane of early apoptotic cells. PI can pass through the impaired membranes of middle and late apoptotic cells. These results indicated that aspirin induced TSC apoptosis when aspirin concentration was 1, 2, or 5 mM, suggesting that a high concentration of aspirin can induce TSC apoptosis. In clinical settings, the blood concentration of aspirin needed to confer anti-inflammatory, antipyretic, and analgesic effects is 150-300 μg/ml, with 180 μg/ml equivalent to 1 mM. According to our results, the recommended clinical dose of aspirin may surpass this critical limit.

The mitochondrial/caspase-3 pathway is a key pathway related to apoptosis. Anti-apoptosis protein Bcl2 and pro-apoptosis protein BAX affect the permeability and potential of cell membranes, which in turn regulate the release of cytochrome C from the mitochondria. Cleaved caspase-3 is a protein of the caspase family that can cause an apoptotic cascade

reaction. Therefore, we observed the changes in levels of Bcl2, BAX, and cleaved caspase-3 in rat TSCs and determined that aspirin-induced apoptosis occurs through the mitochondrial/caspase-3 pathway. Our results from this study are similar to those found in previous research [32-35]. We confirmed our results using an immunofluorescence assay in rat Achilles tendons. Tendons treated with aspirin for 4 weeks demonstrated activation of the caspase-3 pathway. As tendinopathy is a chronic inflammatory disease, patients should take NSAIDs for an extended duration, but long-term aspirin use damages tendons and hampers tendinopathy recovery despite relieving fever and pain.

Previous studies have reported that aspirin affects cells through the JAK1/STAT1 pathway, ERK1/2 activation, and the canonical Wnt/β-catenin pathway [36-39]. Among these pathways, the canonical Wnt/β-catenin signaling pathway is a highly conserved pathway related to cell growth, development, metabolism, and stem cell maintenance. Therefore, we focused on the effect of aspirin on the Wnt/β-catenin pathway. First, we observed the changes in the levels of Wnt/β-catenin proteins, including DKK1, P-GSK-3β/GSK-3β, P-β-catenin/β-catenin, Cyclin D1, and C-myc, in rat TSCs. Then, we confirmed these levels using an immunofluorescence assay in rat Achilles tendons. Under normal conditions, Dickkopf protein family members specifically inhibit canonical Wnt signaling by binding as high-affinity antagonists to lipoprotein receptor-related protein (LRP) co-receptors. After the signal arrives, β-catenin is phosphorylated by the constructive complex (Axin/APC/WTX/CK1a/GSK3β). The P-β-catenin complex is recognized by β-TrCP, and then modified by ubiquitination and degenerated via the proteasome pathway. When Wnt/β-catenin signaling is activated, the constructive complex binds with the membrane receptor Frizzled/LRP complex. The p-β-catenin complex causes a dephosphorylation reaction, and Tcf-4 is released by the repressor protein Groucho. The accumulated β-catenin may affect the downstream effective proteins cyclin D1 and C-myc by combining with Tcf-4. Our results showed that p-GSK-3β (ser9) expression decreased while DKK1 and P-β-catenin expression increased. These changes decreased β-catenin input to the nucleus, and thus decreased expression of the cell cycle proteins Cyclin D1 and C-myc. The results of our immunofluorescence assay agree with the above results, indicating that aspirin has a dose-dependent and time-dependent inhibitory effect on the Wnt/β-catenin pathway.

Li et al. revealed that the canonical Wnt/β-catenin pathway inhibits the apoptosis of tumor cells and mesenchymal stem cells [40] and modulates the differentiation of stem cells [31-32]. Therefore, we next investigated the role of the canonical Wnt/β-catenin pathway in aspirin-induced TSC apoptosis. For this purpose, we pretreated TSCs by adding the Wnt/β-catenin pathway activators wnt3a and LiCl. Wnt3a is a Wnt mimetic, and LiCl is an inhibitor of GSK3. First, we verified the activation effect of Wnt3a and LiCl on the Wnt pathway. Next, we explored whether apoptotic status was attenuated after adding the two activators. As expected, the apoptotic level decreased according to all measurements after the addition of Wnt3a and LiCl. Meanwhile, the expression of the cell cycle proteins Cyclin D1 and C-myc increased. These results suggest that aspirin induces TSC apoptosis through Wnt signaling.

Aspirin prevents thrombus formation by inhibiting COX-1 expression, and exerts anti-inflammatory effects by non-selectively inhibiting COX-2 expression [41]. Tendinopathy is closely related to inflammation. Therefore, we observed the effect of aspirin on COX-2 expression in TSCs. Surprisingly, COX-2 expression dose-dependently increased rather than decreased. However, this was not observed in the time-dependent group. We believe this is because the microenvironment of the TSCs was not inflammatory. To verify whether COX-2 participates in aspirin-induced TSC apoptosis, we added the COX-2 inhibitor NS398 before aspirin was added. We observed that the apoptosis rate of the NS398 group was higher than that of the group without NS398. We hypothesize that elevated COX-2 expression may be a stress-induced reaction that allows the cell to resist apoptosis, though the specific mechanism remains unknown and should be explored in a subsequent study (Fig. 6).

These results suggest restrictions on aspirin dose and length of administration in clinical practice. At concentrations of 1, 2, and 5 mM and a length of administration of 4 weeks, the dose and administration time were within the ranges used in clinical practice.

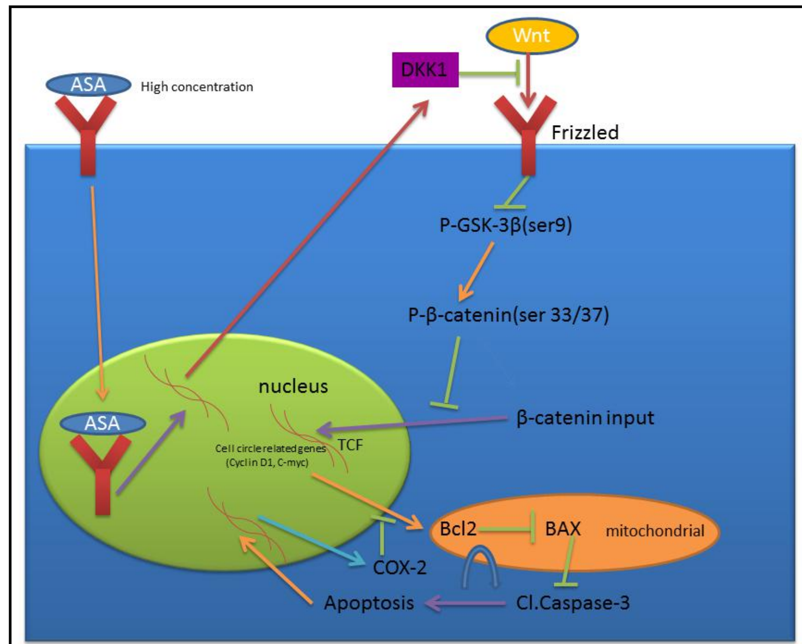

Fig. 6. Schematic illustration of the induction of TSC apoptosis by aspirin through the canonical Wnt/β-catenin pathway. Aspirin increased DKK1 expression, decreased P-GSK-3β (ser9) expression and the input of β-catenin to the nucleus, downregulated Cyclin D1 and C-myc, activated the mitochondrial/caspase-3 pathway and induced the apoptosis of TSCs. Apoptosis in turn caused the upregulation of COX-2, which resulted in negative feedback that inhibited apoptosis.

Therefore, aspirin-induced apoptosis of TSCs indicates that long-term and high-dose aspirin use may have negative effects on the healing of tendinopathy. Administering activators of Wnt signaling may provide new strategies for clinical practice.

There are several limitations to this study. First, we did not expand on the animal tendinopathy model, and the TSCs were not derived from the tendinopathy model. Additionally, we did expand on the inflammatory model of TSCs *in vitro* through LPS or interleukin 1β. Lastly, we did not verify the effect of LiCl or Wnt3a on suppressed cell apoptosis *in vivo*.

Conclusion

This study demonstrated for the first time that aspirin promotes TSC apoptosis via the mitochondrial/caspase-3 pathway and that the inhibition of the Wnt/β-catenin pathway participates in the apoptosis process. This side effect may hinder tendinopathy healing. Molecules that can activate Wnt signaling may be useful targets for treatment. It should be noted that further discussion is needed to determine whether the anti-inflammatory effects of aspirin for treating tendinopathy outweigh the deleterious pro-apoptosis effects.

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Disclosure Statement

No competing financial interests exist among any authors in relation to this submission.

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