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Original Article

Therapeutic effects of asperosaponin VI in rabbit tendon disease

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ABSTARCT

Introduction: This study explored the effects and molecular mechanisms of asperosaponin VI in tendon disease.

Methods: Forty-eight purebred adult male New Zealand white rabbits were randomly divided into the normal group (normal, n = 8); saline group (saline, n = 8) and prostaglandin E2 group (n = 32), which was further divided into four subgroups that were treated with asperosaponin VI doses of 0 mg/kg (model, n = 8), 10 mg/kg (10, n = 8), 20 mg/kg (20, n = 8) and 40 mg/kg (40, n = 8). The expression levels of matrix metallopeptidase 1 (MMP1), metallopeptidase inhibitor 1 (TIMP1), transforming growth factor beta 1 (TGFB1), serpin family E member 1 (SERPINE1), collagen I (COL1), collagen III (COL3) and tenomodulin (TNMD) in Achilles tendon tissue were determined through Western blot analysis. The histopathological changes in tendon tissue were observed by using Masson staining and haematoxylin–eosin staining.

Results: The expression levels of MMP1, TIMP1 and COL3 were higher and those of TGFB1, SERPINE1, COL1 and TNMD were lower in the 0 mg/kg group than in the normal group ($P < 0.05$). Compared with those in the 0 mg/kg group, the levels of MMP1 were lower in the 20 and 40 mg/kg groups. Compared with those in the 0 mg/kg group, the levels of TIMP1 were lower and the levels of TGFB1, COL1 and TNMD were higher in the 10, 20 and 40 mg/kg groups. In addition, compared with those in other groups, the levels of SERPINE1 in the 40 mg/kg group were significantly higher and the levels of COL3 in the 10 and 20 mg/kg groups were significantly lower ($P < 0.05$). Fibrous tissue arrangements and structures in the 40 mg/kg group were similar to those in the control group.

Conclusion: The effects of asperosaponin VI on injured tendons mainly involve eliminating inflammation, restoring balance to extracellular matrix collagen metabolism and inducing tendon cell proliferation. Asperosaponin VI is likely to be an ideal drug for the prevention and treatment of tendon disease.

1. Introduction

Tendon disease is a common orthopaedic condition that often occurs in athletes and the elderly and is mainly caused by degenerative changes in tendons caused by long-term overuse [1]. Recovery from this condition has a long course and can be hampered by the poor self-repair capability of tendons [2]. More than 30 million tendon- or ligament-related surgeries occur worldwide every year, thus exerting a massive economic and social burden [3]. In the United States and the European Union, more than €150 billion (US $181.8 billion) is spent yearly on tendon disease surgeries [3].

Trauma, strain, metabolic disorders and other factors accelerate dynamic and static imbalances in tendons and bones, thereby leading to tendon disease [4]. Physiotherapy oral analgesia and infiltrations [5], extracorporeal shock waves [6], aspirin, fluoroquinolones [7–9], local steroid injection and tuberculosis osteotomy are used to treat tendon lesions [10,11]. Therapeutic mechanisms have been explored at the molecular level and include exocrine body and tendon stem cell differentiation and synovial multifunctional cell repair [12–14]. Matrix metallopeptidase 3 and metallopeptidase inhibitor 2 (formerly known as metalloproteinase inhibitor 2) gene variants may cause susceptibility to chronic Achilles tendon diseases or mechanical stress effects under

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different loads [15,16]. Studies have shown that interferon, NF-kappa B and signal transducer and activator of transcription 6 (STAT6)—the downstream targets of M1 and M2 macrophage polarisation—are activated in the early stages of tendon disease [17]. M2 polarisation is upregulated in the late stages of tendon disease, thus activating STAT6 downstream [18]. However, effective drugs and treatments for tendon disease remain lacking. Therefore, studies on tendon disease at the molecular level should be helpful for developing new treatment strategies.

Radix Dipsaci, which contains asperosaponin VI as an important active compound, has analgesic and anti-inflammatory properties. Asperosaponin VI possesses neuroprotective, myocardial protective, antiosteoporosis, liver protective and lipid-lowering effects that are consistent with tonifying the liver and kidney [6], strengthening muscles and bones and repairing bones. Previous studies have demonstrated that asperosaponin VI improves cell proliferation [19–23], tendon healing [24] and anti-inflammatory responses [25]; slows apoptosis and affects abnormal stem cell differentiation by regulating transforming growth factor beta 1 (TGFB1)/Smads, BCL2-associated X apoptosis regulator (BAX), caspase 3, hypoxia inducible factor 1 subunit alpha/vascular endothelial growth factors [26], bone morphogenetic proteins, alkaline phosphatase, bone gamma-carboxyglutamate protein (formerly called osteocalcin) and runt-related transcription factors [27]. In recent years, studies have considered the proliferation of tendon cells and the differentiation of stem cell tendon systems to be closely associated with the repair of tendon disease [28,29]. Unfortunately, only a few reports on asperosaponin VI for the treatment of tendon diseases exist.

In this study, we used a rabbit model of tendon disease and performed Western blot analysis to detect the expression levels of matrix metallopeptidase 1 (MMP1), metallopeptidase inhibitor 1 (TIMP1), TGFB1, serpin family E member 1 (SERPINE1), collagen I (COL1), collagen 3 (COL3) and tenomodulin (TNMD) in Achilles tendon tissue. Histopathological changes in tendon tissue were observed via Masson staining and haematoxylin–eosin staining. The purpose of this work is to explore the effect of asperosaponin VI in the treatment of tendon disease to provide a theoretical basis and practical guidance for the use of asperosaponin VI as a potential drug for tendon disease. We hypothesise that asperosaponin VI is likely to be an ideal drug for the prevention and treatment of tendon disease.

2. Materials & methods

2.1. Animal care

This animal experiment was approved by the Animal Ethics Committee of Chengdu Institute of Sports (approval no.: Adult Ethics [2020] 21). Purebred adult male New Zealand white rabbits ($n = 48$, mean body mass: 2.04 ± 0.16 kg) were reared in the animal room of the Sichuan Key Laboratory of Sports Medicine. The rabbits were housed with one animal per cage and given national standard rodent feed (Chengdu, SCXK [Chuan] 2013–24, Dashuo Biotechnology Co., Ltd.). The animal room was ventilated and kept dry with a relative humidity of 55%–70% and room temperature of 20 °C-25 °C. All experimental animals successfully completed the experimental cycle. No instances of abnormal body mass, Achilles tendon redness, swelling, pus or other diseases were observed.

2.2. Main reagent

Asperosaponin VI (American Chemical Abstract CAS No.: 39524-08-8, Shanghai Yuanye Biotechnology Co., Ltd) with high-performance liquid chromatography detection purity ≥98% (batch number: Z18M10L83256) was used. Prostaglandin E2 was purchased from Ron Company (Shanghai).

2.3. Animal groupings

The rabbits were fed adaptively for 1 week, after which they were randomly allocated to the prostaglandin E2 group ($n = 32$), saline group ($n = 8$) [30] or normal group ($n = 8$) through a stochastic numerical method. Each rabbit in the prostaglandin E2 group was fixed on an operating table in a prone position and then injected with 300 ng of prostaglandin E2 at 2.0 cm proximally to the left Achilles tendon's insertion into the calcaneus once a week for 4 weeks [31]. Each rabbit in the saline group was fixed on the operating table in a prone position and injected with 0.2 mL of saline at 2.0 cm proximally to the left Achilles tendon's insertion into the calcaneus once a week for 4 weeks. The rabbits in the normal group did not receive any injections.

2.4. Intervention treatment

The rabbits in the model group were subdivided into the 0, 10, 20 and 40 mg/kg asperosaponin VI groups through the random allocation of eight rabbits to each subgroup. The rabbits in the model group were given intraperitoneal injections of 10, 20 or 40 mg/kg asperosaponin VI dissolved in saline or saline only (0 mg/kg asperosaponin VI) once a day for 4 weeks. The rabbits were fasted and were not given water for 24 h after the last administration. The rabbits were weighed and then killed via air embolisation.

2.5. Ultrasonic inspection

Given the superficial location of tendons, musculoskeletal system ultrasonography is the most suitable diagnostic tool and is generally the initial imaging modality for tendon disorders [32]. At the end of the 4 weeks of treatment, two rabbits in each group were randomly selected for musculoskeletal ultrasound examination (Siemens S2000), including musculoskeletal ultrasonography and musculoskeletal blood flow characterisation. Echo intensity, Achilles tendon thickness, blood flow and inflammatory response were observed.

2.6. Western blot analysis

Cells and tissue were collected. Tendon tissue was ground and used for the determination of total protein levels with a bicinchoninic acid kit. SDS-PAGE electrophoresis, membrane transfer and immunohybridisation were performed. The levels of MMP1, TIMP1, TGFB1, SERPINE1 (formerly called plasminogen activator inhibitor 1), COL1, COL3 and TNMD in the tissue were detected through Western blot analysis.

2.7. Masson staining

Tissues from the lower left extremities of all animals were collected and rinsed twice with phosphate-buffered saline. They were fixed in 40 g/L paraformaldehyde for 24 h, embedded in paraffin, sectioned longitudinally and stained (Masson). Longitudinal 4–6 μm sections were analysed under a light microscope by professional pathologists. Tendon fibre morphology was examined to verify vascular proliferation, inflammatory cell infiltration and other lesions and was compared between groups.

2.8. Haematoxylin–eosin staining

Tissues from the Achilles tendon of the left lower limb were collected, fixed with formaldehyde and embedded in paraffin. Longitudinal 4–6 μm sections were stained with haematoxylin–eosin. Tendon fibre morphology, nuclei, vascular proliferation and inflammatory cell infiltration were observed under a light microscope. Changes in fibrous tissue arrangement, nuclear morphological density, inflammatory cell infiltration degree and neovascularisation were assessed under a light microscope in accordance with the Chen Lei semiquantitative scoring standard (in which 0 is normal and 3 is severely injured) [33]. The scoring criteria are shown in Table 1.

2.9. Statistical analysis

GraphPad Prism (version 8, GraphPad Software, Inc.) was used for data processing and mapping. Data were reported as mean ± SD. Data with homogeneity of variance were analysed through one-way analysis of variance (ANOVA) and data with uneven variance were analysed with Brown–Forsythe and Welch ANOVA with multiple comparisons ($\alpha = 0.05$).

3. Results

3.1. Effects of asperosaponin VI on the ultrastructure of the Achilles tendon

The normal group had tendons with uniform and continuous echo intensity and clear boundaries and did not exhibit abnormal blood flow. In the model group, the tendons were thick and abnormal and many inhomogeneous echo masses were observed at 2 cm proximally to the left Achilles tendon's insertion into the calcaneus. Moreover, the boundaries of collagenous fibres were unclear, the echo signal of the surrounding fascia was enhanced and blood flow was abundant. In the saline group, homogeneous echoes were present and no abnormal blood flow was observed. In the 10 mg/kg asperosaponin VI group, the echo intensity of the surrounding fascia was enhanced, blood flow was abundant, the tendons had thickened and inflammatory cell infiltration was observed. In the 20 and 40 mg/kg asperosaponin VI groups, the echo intensity was uniform, tendon thickening was not observed and the boundaries were clear. The two groups had similar characteristics (Fig. 1).

3.2. Protein expression

The levels of MMP1, TIMP1 and COL3 were higher in the model group than in the normal group ($P < 0.05$). The levels of TGFB1, SERPINE1, COL1 and TNMD in the model group were downregulated ($P < 0.05$) compared with those in the normal group. MMP1 was downregulated in the 20 and 40 mg/kg groups relative to in the model group ($P < 0.05$). TGFB1, COL1 and TNMD were upregulated in the 10, 20 and 40 mg/kg groups ($P < 0.05$). SERPINE1 was significantly higher in the 40 mg/kg group ($P < 0.05$) than in the model group. COL3 expression was downregulated in the 10 and 20 mg/kg groups compared with that in the model group ($P < 0.05$; Fig. 2).

3.3. Masson staining and haematoxylin–eosin staining

Muscle fibrous tissues were clearly visualised through Masson staining and haematoxylin–eosin staining. The muscle fibres were neatly arranged and strongly stained in the normal group. In the model group, no clear fibre arrangement structure was observed and the tendon fibres were disordered with a wavy arrangement. The tissue structure was incomplete with abnormal neovascularisation proliferation, inflammatory cell infiltration, round nuclei, elevated cell densities and adipoid changes. In the 40 mg/kg group, the fibrous tissue showed slight changes. The tendon fibres were wavy but continuous and arranged in an orderly manner, and the cell densities were normal. No clear inflammatory cell infiltration was observed. In the 20 mg/kg group, the fibrous tissue was slightly disordered, the fibres were intact but arranged with a wave-like pattern and inflammatory cell infiltration was evident. In the 10 mg/kg group, the fibrous tissue was loose and slightly broken and the collagen fibres were short, wavy, disordered and curled. The nuclei were deformed, inflammatory cell infiltration was evident and cell densities were elevated (Fig. 3).

The tendon damage scores in the 10, 20 and 40 mg/kg asperosaponin VI groups decreased successively and were significantly lower than those in the model group ($P < 0.05$; Fig. 4).

4. Discussion

The purpose of this study is to explore the effect of asperosaponin VI in the treatment of tendon disease to provide a theoretical basis and practical guidance for the use of asperosaponin VI as a potential drug for tendon disease. The research hypothesis of this work was verified.

4.1. Therapeutic effects of asperosaponin VI on Achilles tendinopathy

In rabbits administered with the highest dose of asperosaponin VI (40 mg/kg), the arrangement and distribution of fibrous tissue were similar to those of normal tendon tissue and no clear inflammatory cell infiltration or vascular dysplasia was observed. In addition, no significant difference between the saline group and normal group was observed, indicating that the injection of saline had no effect on normal tendon tissue. In lesions, collagen fibre injury and neovascularisation co-occur during tendon tissue remodelling until the balance of cell matrix remodelling is broken [12]. Tendon healing is completed mainly through cells and

Table 1
Semi-quantitative rating scale for tendon healing assessment.

Index	0	1	2	3
Fiber structure	Continuous, long fiber	Slight fracture	Moderate fracture	Serious fracture
Fiber arrangement	Compact, parallel	Slightly loose, wavy	Moderately loose, wavy, crossed	No recognizable form
Circular core	Long spindle cell	Slightly round	Relatively round	Very round
Inflammation (area of infiltration of inflammatory cells)	< 10% (Extracellular matrix)	10%–20% (Extracellular matrix)	20%–30% (Extracellular matrix)	> 30% (Extracellular matrix)
Neovascularization (infiltration area)	< 10% (Extracellular matrix)	10%–20% (Extracellular matrix)	20%–30% (Extracellular matrix)	> 30% (Extracellular matrix)
Cell density	Normal	A little	General	A lot

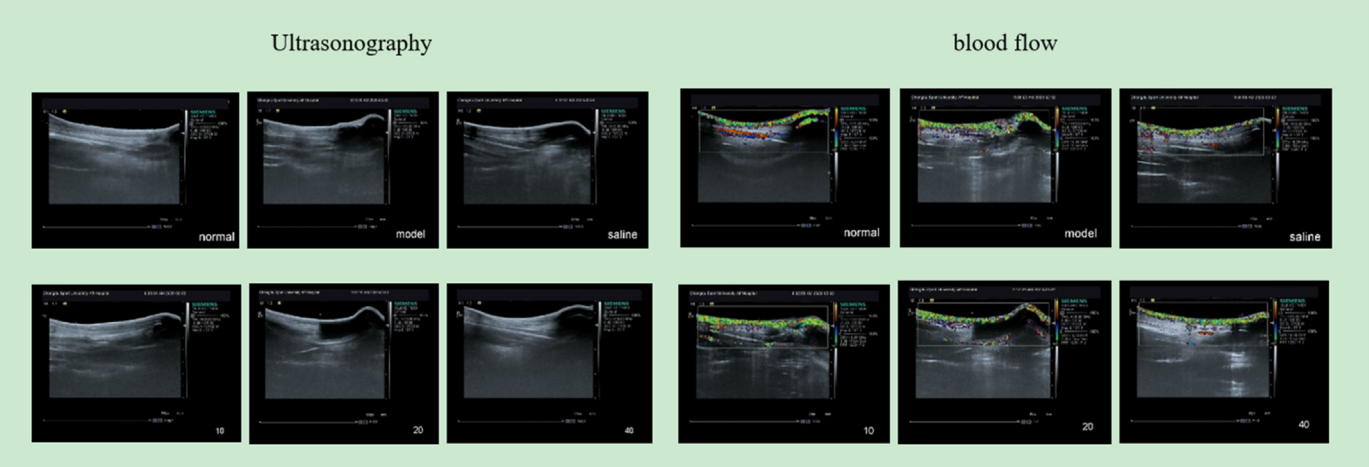

Fig. 1. Effects of asperosaponin VI on the ultrastructure of Achilles tendons in different groups. Abnormal echo and abnormal blood flow signal in the model group. In the 10 mg/kg group, the echo mass was not uniform, the echo of the surrounding fascia was enhanced, the tendon was clearly thickened and blood flow was abundant. The inflammatory response in the 10 mg/kg group was greater than that in the 20 and 40 mg/kg groups. In the 20 and 40 mg/kg groups, the echo intensity was uniform, tendon thickening was unclear and the boundary was clear. The two groups had similar ultrasonic manifestations.

extracellular matrix repair processes, which are divided into endogenous and exogenous healing processes. Inflammation is central to successful healing. Aberrant, excessive or insufficient inflammation has profound effects on tendon healing [34]. After injury, tissue repair and scar formation begin and a process that includes tissue inflammation, cell proliferation and extracellular matrix remodelling occurs [35]. Our findings provide support that asperosaponin VI repairs tendinopathy through these mechanisms. Asperosaponin VI has been speculated to repair and protect damaged tendons in tendon disease by eliminating inflammation, inducing cell proliferation, promoting collagen repair and maximising the expression of COL1 and proteoglycan, thus accelerating endogenous healing [36].

4.2. Molecular mechanism of asperosaponin VI in repairing achilles tendinopathy

This study showed that the levels of MMP1 and TIMP1 in the group that was given the highest dose of xxx (40 mg/kg) were closest to normal levels. We speculate that asperosaponin VI decreases apoptosis and accelerates the extracellular matrix remodelling of injured tendons by restoring the metabolic balance of MMP1/TIMP1. Matrix metallopeptidases degrade the extracellular matrix, induce intracellular calcium release and lead to apoptosis [37]. MMP1 regulates metabolism and extracellular matrix remodelling in tendon tissue and decomposes COL1 and damaged or necrotic tendon tissue. TIMP1 is an antagonist of MMP1. Under pathological conditions, MMP1 levels increase sharply at a faster rate than TIMP1 levels (i.e. the MMP1/TIMP1 ratio increases), thus placing the matrix in a state of metabolic imbalance with net collagen fibre degradation and leading to tendon rupture or tendon disease [38]. Some studies have demonstrated that asperosaponin VI decreases the production of reactive oxygen species and inhibits the disruption of mitochondrial membrane potential and endothelial cell apoptosis by regulating the expression of Bcl-2, Bax and caspase 3 [39,40].

The expression of COL1 and COL3 plays an important role in tendon injury and repair; moreover, the expression of COL1 and TNMD is positively correlated with the level of tendon repair. The expression of COL1 was upregulated in the 10, 20 and 40 mg/kg asperosaponin VI groups after 4 weeks of treatment. In addition, the 40 mg/kg treatment showed the optimal effect in promoting endogenous tendon repair. The expression of COL3 in the 10 and 20 mg/kg asperosaponin VI groups was lower than that in the 40 mg/kg group but was still higher than that in the normal group. TNMD is a member of the type II transmembrane glycoprotein family [41]. Its C-terminus contains an antiangiogenic region, which is a component of proteoglycans and glycoproteins in the tendon extracellular matrix. TNMD's high expression in tendon tissue is considered to be an important factor in the proliferation and maturation of tendon cells. In addition, it has particular importance as a marker of the differentiation of stem cell into tendon cells [42]. The expression of TNMD in the 10, 20 and 40 mg/kg asperosaponin VI groups was upregulated in a dose-dependent manner, thus suggesting that asperosaponin VI promotes extracellular matrix glycoprotein remodelling and collagen repair in tendons.

This study suggests that asperosaponin VI may promote extracellular matrix collagen remodelling, tendon cell proliferation and local anti-inflammatory effects via the TGFB1 pathway. Given that TBGF1 may have a two-way regulatory effect—i.e. it not only affects exogenous repair (tendon adhesion and tissue scar formation) but also promotes tendon healing and other endogenous repair processes—its role in tendon repair is complex and its effects must be further explored.

TGFB1 is an effective index of tendon repair. The upregulated expression of TGFB1 promotes tendon healing and enhances strength [43–45]. TGFB is produced in human rotator cuff tendon cells and promotes tendon repair [46]. TGFB also activates Ras/ERK and consequently promotes DNA synthesis and cell proliferation through type I receptors or indirectly promotes collagen synthesis [47,48]. TGFB1 has also been suggested to promote the decomposition of fibroblasts by cooperating with platelet-derived growth factors, insulin-like growth factor 1 (IGF1), fibroblast growth factors, epidermal growth factors and vascular endothelial growth factors [49]. TGFB participates in tendon repair and healing. The high expression of TGFB1, IGF1 and the proliferating cell nuclear antigen gene promotes collagen synthesis, tendon cell proliferation and tendon regeneration. TGFB inhibits the expression of MMP1 in human epidermal fibroblasts and the epidermal keratinised cell line A-5 [50]. TGFB upregulates the expression of an inhibitor of metallopeptidase mRNA in human peritoneal mesothelial cells [51]. TGFB also promotes tendon repair by activating Smad and mitogen-activated protein kinase pathways and by stimulating the

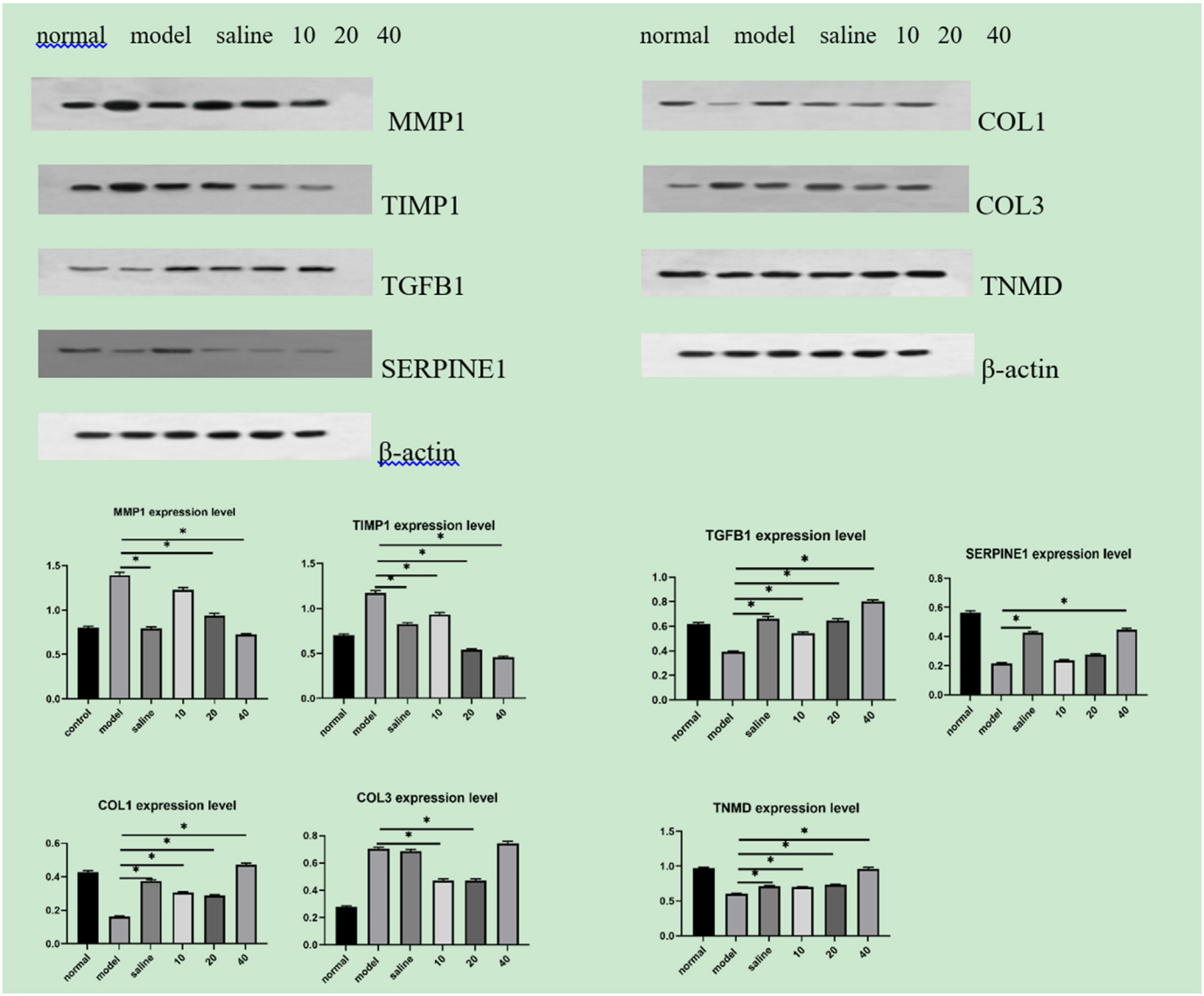

Fig. 2. Protein levels of MMP1, TIMP1, TGFB1, SERPINE1, COL1, COL3 and TNMD in different groups determined via Western blot analysis. Compared with the model group, *P < 0.05.

production of AP-1 transcription factors and other downstream targets, thus inhibiting the expression of matrix metallopeptidases/metallopeptidase inhibitors [52]. However, some studies have found that in young sheep, TGFB1 is not highly expressed in tendon repair; this phenomenon results in a disordered extracellular matrix through the direct inhibition of proteoglycan expression [53,54]. Farhat suggested that TGFB1 has multiple biological effects in promoting collagen expression, perturbing the balance of the extracellular matrix and causing fibrosis in tendons [55]. Although inhibiting the expression of TGFB1 may not necessarily improve the mechanical properties of tendons, it can decrease tendon adhesion and tissue scar formation [56].

Asperosaponin VI may increase the expression of SERPINE1 in a dose-dependent manner. Farhat found that TGFB1 directly upregulates MMP2 and SERPINE1 [57]. Abnormal components in the extracellular matrix of tendons are the most important factors leading to fibrosis [58]. SERPINE1, the main inhibitor of the urokinase plasminogen activation system, causes cell migration and infiltration by interfering with cell adhesion and promoting basement membrane degradation [58]. The high expression of SERPINE1 in scar fibroblasts results in fibrosis and scar formation by decreasing fibrin degradation and leading to the deposition of large amounts of collagen in the extracellular matrix [59]. SERPINE1 may provide a potential therapeutic target for tendon remodelling [60]. However, the further consideration of its effects is necessary to delay or prevent the occurrence and progression of fibrosis.

4.3. Limitations

Although we preliminarily concluded that asperosaponin VI could effectively inhibit inflammation, promote cell proliferation and collagen remodelling and may be an ideal drug for the prevention and treatment of tendinopathy, its underlying mechanism still needs to be further explored. Previous studies have illustrated that asperosaponin VI can promote wound repair by enhancing angiogenesis and cell proliferation and migration. However, tendon tissue is hypovascular tissue, and Achilles tendons heal slower than other tissues. Angiogenesis is a key factor in tissue repair, and vascular supply plays an important role in primary tendon healing, especially in the early stages of healing. The Hif-1 α protein stabilises and activates the expression of several genes critical for angiogenesis. We will investigate whether the mechanism of

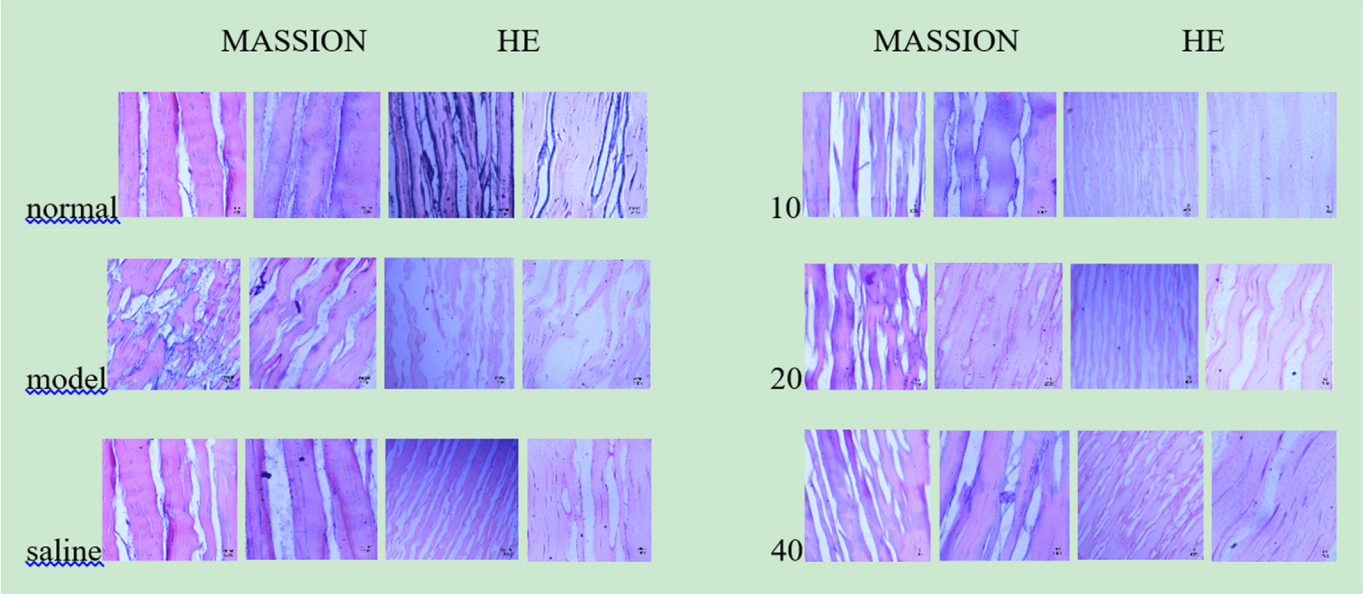

Fig. 3. Effects of asperosaponin VI on pathological changes of different groups: Masson staining (100 × , 200 ×) and haematoxylin–eosin staining (40 × , 200 ×).

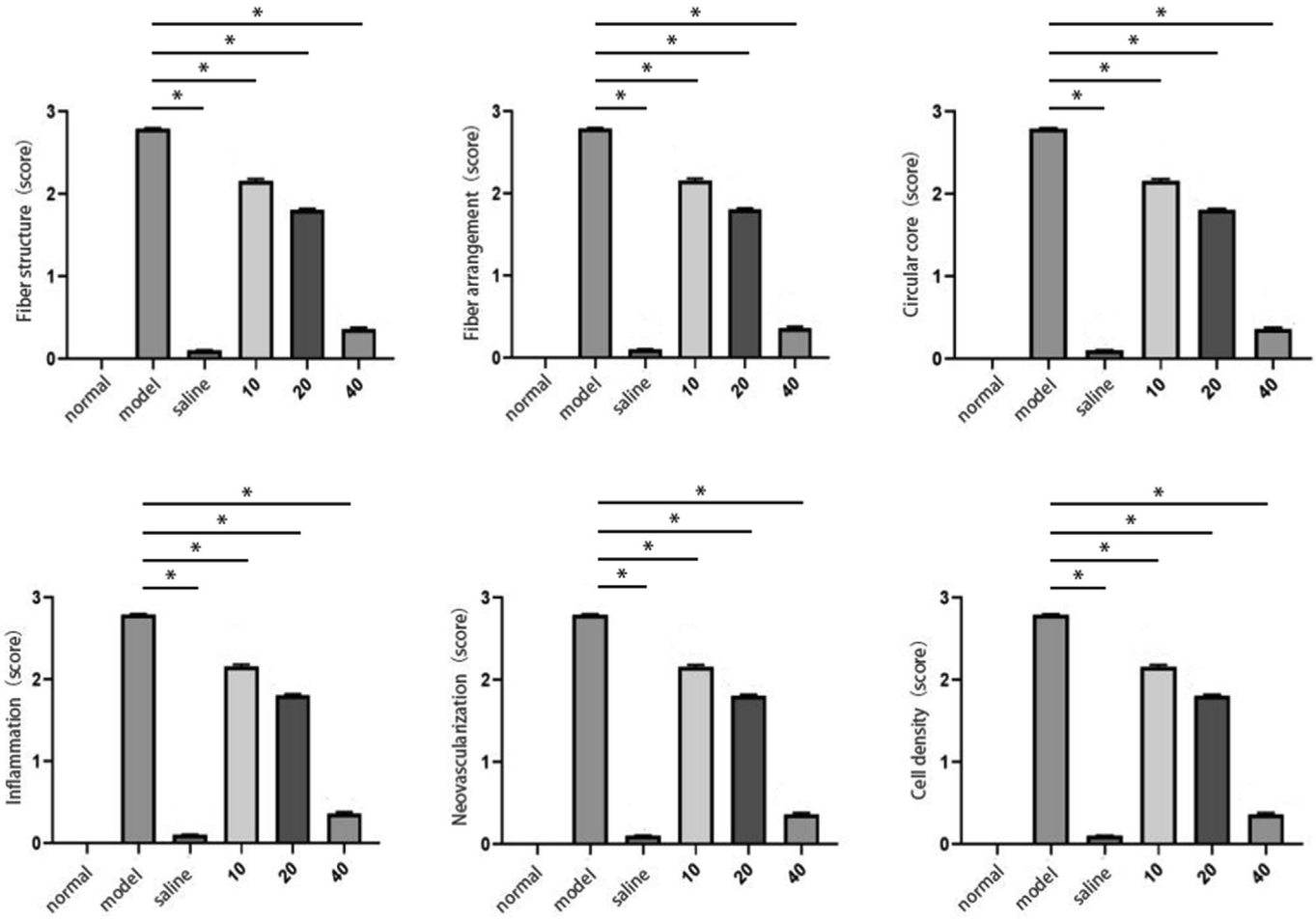

Fig. 4. Effects of asperosaponin VI on the evaluation score of tendon healing. Compared with the model group, *P < 0.05.

asperosaponin VI treatment for tendinopathy is related to the upregulation of hypoxia-inducible factor 1α/vascular endothelial growth factor signal.

5. Conclusion

The effects of asperosaponin VI on injured tendons mainly involve eliminating inflammation, restoring the balance of extracellular matrix collagen metabolism and inducing tendon cell proliferation. Asperosaponin VI balances the MMP1/TIMP1 ratio and promotes the expression of TNMD, TGFB1 and SERPINE1. Asperosaponin VI is likely to be an ideal drug for the prevention and treatment of tendon disease.

Author contributions

All authors planned the experimental design together. Kun Wang was responsible for collecting and analyzing data, and writing the preliminary draft, Benxiang He reviewed and revised.

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Data availability

Any of the information is supplied as supplementary file or can be obtained from the author on request.

Declaration of competing interest

The authors declare no competing interests.

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