Original Paper

Thermal Preconditioning May Prevent Tendon Adhesion by Up-Regulating HSP72 in Rats

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Key Words

Thermal preconditioning • Heat shock protein 72 mRNA and protein expressions • Tendon adhesion • Rat model • Tendon surgery

Abstract

Background/Aims: The study aims to determine the effects of thermal preconditioning on tendon adhesion by regulating the expression of heat shock protein 72 (HSP72) in rat models. **Methods:** Sixty male Wistar rats were collected and randomly assigned into the thermal preconditioning and control groups. During the 4th and 8th weeks following surgery, 15 rats were sacrificed in each group, respectively, and their tendon adhesion was observed and evaluated. Biomechanical test was performed to measure the tensile strength and gliding distance of tendon. Hematoxylin-eosin (HE) was used to observe the morphological structure of the tendons. Histochemical staining, quantitative real-time polymerase chain reaction (qPCR) and Western blotting were used to detect the HSP72, fibroblast growth factor-2 (FGF-2), fibroblast growth factor receptor-1 (FGFR-1), β -catenin, epithelial cell adhesion molecule (EPCAM), Tenomodulin and scleraxis protein expressions. Pearson correlation analysis was applied to analyze the correlation between HSP72 expression and tendon adhesion. **Results:** At the 4th week after surgery, we found no differences in the tendon adhesion scores or mRNA and protein expressions of HSP72 between the thermal preconditioning and control groups. However, after the 8th week after surgery, the thermal preconditioning group had a lower tendon adhesion score and higher mRNA and protein expressions of HSP72 than the control group. During the same period, we found longer gliding distance and higher expression levels of FGF-2, FGFR-1, β -catenin, Tenomodulin and scleraxis, but lower EPCAM expression in the thermal preconditioning group. Pearson correlation analysis indicated that HSP72 mRNA and protein expression levels were negatively correlated with tendon adhesion. **Conclusions:** These findings provide evidence that thermal preconditioning may alleviate tendon adhesions via upregulation of HSP72 expression.

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Introduction

Tendon laceration is a common physical injury that is often caused by accidental trauma. The restoration of its full function following injury depends highly on timely surgical treatment [1]. During the treatment process, tendon adhesion is necessary for loss of restoring function. However, excessive tendon adhesion may negatively affect the gliding function of the tendons, which is a major clinical complication following tendon surgical treatment [2]. Repaired tendons may develop complications, such as progressive fibrosis, which results in cause adhesion formation and leads to a reduced range of motion [3]. Previous work has investigated the different processes involved in tendon healing, such as inflammation, proliferation, collagen synthesis, vascularization, and apoptosis [4]. It is difficult to prevent postoperative adhesion in tendon lacerations [1]. Adhesion prevention includes postoperative treatment strategies and pharmacological interventions. Anti-adhesive agents, such as hyaluronic acid, and anti-inflammatory drugs like corticosteroids, have been administered with reduced adhesion [5]. A study reported that the regulation of heat shock protein 72 (HSP72) by heat and mechanical stress in human fibroblasts involves a nuclear translocation mechanism [6].

HSP72 is a stress-inducible protein member of the heat shock protein 70 family. HSP70 are an important component of the cell's machinery in protein folding that help protect the cell during stress conditions. HSP72 functions with other molecular chaperones to mediate protein folding and stabilize pre-existing proteins against denaturation [7]. Additionally, HSP72 also a wide variety of roles in an important role in intracellular protein transport, cytoskeletal architecture, mutation masking, intracellular redox homeostasis and protection against spontaneous or induced programmed cell death [8]. HSP72 has been reported to be synthesized as a cytoprotective response to cellular stress, and the up-regulation of HSP72 by heat and mechanical stress is a response observed in human fibroblasts that involves its nuclear translocation [6]. Furthermore, fibroblast growth factor 2 (FGF-2) was found to accelerate the initial tendon-to-bone remodeling, and its counterpart receptor, fibroblast growth factor receptor-1 (FGFR-1) plays a multifunctional role in bone formation and callus remodeling [9, 10]. A previous study reported that β -catenin might be involved in bone healing after fracture, especially with respect to chondrogenesis and endochondral ossification [11]. Hiraga et al. demonstrated that epithelial cell adhesion molecule (EPCAM) may play an important role in cell adhesion [12]. Additionally, EPCAM expression is related to proliferative activity and is involved in neoplastic transformation; also, regulation of EPCAM expression can affect cell proliferation, migration and invasion by increasing E-cadherin mediated cell-cell adhesion [13]. In 2002, it was shown that thermal preconditioning prevents posttendon adhesions and inflammation in a rabbit model [14]. As an existing study showed that thermal preconditioning could prevent a disuse-induced fiber type transformation by inducing HSP72 [15], we hypothesized that thermal preconditioning could also prevent tendon adhesions by inducing HSP72. In the present study, we aimed to investigate the effects of thermal preconditioning on tendon adhesion and its correlation with FGF-2, FGFR-1, β -catenin, Tenomodulin, and EPCAM in tendon tissues.

Methods and Materials

Ethics statement

All animals were reared and treated in strict accordance with the US Guidelines for the Management and Use of Laboratory Animals [16]. All procedures performed in the study have received the approval of the Laboratory Animal Ethics Committee in Zhongnan Hospital of Wuhan University.

Experimental animals and grouping

Male Wistar rats (n = 60, 10 weeks of age, 350 - 450 g of body weight) were purchased from Hubei Research Center of Laboratory Animals (Wuhan, China). The rats received adaptive feeding in natural light

were kept in conditions of 23 ~ 25°C with 45 – 60% humidity for one week. All 60 rats were randomly divided into the thermal preconditioning group and control group (with no thermal preconditioning). Each group consisted of 30 rats, half of which were kept for the testing on the 4th and 8th weeks after surgery. The rats in both groups were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (45 mg/kg). Rats belonging to the thermal preconditioning group were maintained in a 45°C incubator until their rectal temperature reached 41.5 ± 0.5°C. This was detected by a clinical thermometer for rectal use; then, they sustained the temperature for 15 min. The rats in the control group did not receive any thermal preconditioning. All rats recovered for 24 h at room temperature. After their resting period, none of the rats died or showed signs of lethargy or fatigue.

Tendon surgery and tendon adhesion evaluation

Twenty-four hours after thermal preconditioning, the rats from both groups were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (25 mg/kg) and then fixed horizontally on the operating table. The rats' left hind limb was then shaved and disinfected with iodine. A "C" shape incision was made into the skin of the Achilles tendon to expose the thick and thin tendons. The thick tendon was removed first, followed by a cut made into the middle part of thick Achilles tendon. Non-destructive stitching was applied to tie the two ends of the thick and thin Achilles tendons and skin wounds. The incision was covered with sterile gauze, and the animals could recover. During the 4th week after surgery, all rats wounds healed well without inflammation, infection or death, and the 15 rats in both groups at the 4th week after surgery in both groups were sacrificed. The entire Achilles tendon complex was harvested after killing off the rats for biomechanical testing. The same procedure was done to remain 15 rats during the 8th week after surgery. The remaining 15 rats in both groups were sacrificed. Next, the entire Achilles tendon complex was removed at the 4th and 8th weeks after surgery for biomechanical testing. At the same time, the double-blind method was used to detect postoperative tendon adhesion and tendon gliding function. The tendon adhesion was evaluated according to the following scoring method [17]: 0 points, no adhesion and the tendon glides freely without damage; 1 to 2 points, mild adhesion with lacertus adhering to the surrounding tissues on the tendons and tendon gliding is mild; 3 to 4 points, moderate adhesion with distributed lacertus adhesion to the surrounding tissues and significantly limited tendon gliding; and 5 to 6 points, many lacertus adhering to the surrounding tissues, difficulty separating the tendons and from surrounding tissues and no gliding function.

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

Total RNA was extracted from 0.1 g of rat tendon tissue using an RNA extraction kit (Beijing Tian Enze Gene Technology Co., Ltd, Beijing, China). The reverse transcription kit was purchased from Hangzhou Bioer Technology Co., Ltd (Hangzhou, China). qRT-PCR was conducted according to the manufacturer's instructions using an Applied Biosystems® 7500 PCR System (Applied Biosystems, Inc., CA, USA). The

Table 1. Primer sequences used for qRT-PCR. qRT-PCR, quantitative real-time polymerase chain reaction; HSP72, heat shock protein 72; FGF-2, fibroblast growth factor-2; FGFR-1, fibroblast growth factor receptor-1; EPCAM, epithelial cell adhesion molecule; GAPDH, glyceraldehyde phosphate dehydrogenase

Gene	Direction	Sequence (5' - 3')
HSP72	Forward	GGCTGACAAGAAGAAGGTGCT
	Reverse	CCTCTAATCCACCTCCTCGAT
FGF-2	Forward	CTGGCTATGAAGGAAGATGGA
	Reverse	TGCCCAGTTTCGTTTCAGTG
FGFR-1	Forward	CTTGCCGTATGTCCAGATCC
	Reverse	CCTTACACACATACTCCCCGC
β-catenin	Forward	TTGAAAATCCAGCGTGGACA
	Reverse	TCGAGTCATTGCATACTGTC
EPCAM	Forward	CGCAGCTCAGGAAGAATGTG
	Reverse	TGAAGTACACTGGCATTGACG
Tenomodulin	Forward	GTGGTCCCACAAGTGAAGGT
	Reverse	GTCTTCCTCGCTTGCTTGTC
Scleraxis	Forward	AACACGGCCTTCACTGCGCTG
	Reverse	CAGTAGCACGTTGCCACGGTG
GAPDH	Forward	GTCCGGTGTGAACGGATT
	Reverse	ACTCCACGACGTACTCAGC

primer sequences used for qRT-PCR are shown in Table 1. The PCR kit was purchased from Bio-Rad Inc. (Hercules, CA, USA). The PCR reaction procedures were performed as follows: 94°C pre-denaturation for 5 minutes, 94°C denaturation for 30 seconds, 58°C annealing for 30 seconds, and 72°C extension for 1 minutes. The denaturation - annealing - extension procedure was performed for 40 cycles. Differences in the mRNA expression between the thermal preconditioning and control groups were analyzed by the $2^{-\Delta\Delta Ct}$ method. The threshold cycle (CT) values of each reaction tube were obtained after the reaction, representing the number of amplification cycles when the real-time fluorescence intensity of the reaction reached the set threshold value. Multiple proportions for the relationship of the target gene expressions between thermal preconditioning and control groups were revealed by the $2^{-\Delta\Delta Ct}$ method, and the formula was used as follows: $\Delta\Delta Ct = \Delta Ct_{\text{thermal preconditioning group}} - \Delta Ct_{\text{control group}}$ ($\Delta Ct = C_{t \text{ HSP72}} - C_{t \text{ GAPDH}}$) [12].

Western blotting

The rat tendon tissue (0.1 g) was mechanically broken down and added to 0.5 mL phosphate-buffered saline (PBS) solution (1.9 mmol potassium dihydrogen phosphate, 8.1 mmol potassium hydrogen phosphate, 75 mmol sodium chloride, pH 7.4). The cell suspension was subject to an ultrasonic disruptor (Ningbo Scientz Biological Technology Co., LTD., Ningbo, China) to disrupt the cells for a total duration of 5 minutes (1 second pulse with a 1-second inter-pulse interval). The samples were centrifuged at 10,000 g for 10 minutes to remove the cellular debris. The total protein concentration of the supernatant (30%) was determined using the Bradford method. The results of the Bradford assay were used to normalize the concentration of the protein samples. The proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins in the polyacrylamide gel were transferred onto a polyvinylidene fluoride (PVDF) membrane through a Semi-Dry Blotter (Bio-Rad, Laboratories, Hercules, CA, USA). The PVDF membrane was first soaked in methanol for 15 seconds; then, the polyacrylamide gel and PVDF membrane were soaked in buffer (25 mmol Tris, 190 mmol glycine and 20% (v/v) methanol) for 10 min at 17 V for 30 min. The transferred PVDF membranes were blocked at room temperature for 2 hour in 6% (m/v) skim milk powder (in PBS solution) and then washed for three times, three minutes each with a PBST buffer solution [PBS solution with a final concentration of 0.1% (w/v) Tween - 20]. Samples were incubated at room temperature for 1 hour with rabbit anti-human monoclonal antibodies HSP72, FGFR-1, EPCAM, β -catenin, Tenomodulin, scleraxis, and GAPDH (all from Zhongshan Golden Bridge Biotechnology Co., LTD., Beijing, China), which was followed by washing with PBST buffer five times three minutes each time. Next, the samples were incubated at room temperature for 1 hour with a horseradish peroxidase (HRP) labeled sheep anti-rabbit IgG (Beijing Zhongshan Golden Bridge Biotechnology Co., LTD., Beijing, China), which was followed by washing with PBST buffer five times for 3 min each time. The HRP substrates (Bio - Rad) were used to color the target proteins.

Image - Pro Plus was used to detect the gray value of the target strip area in the Western blotting results. The ratio of target protein/internal reference protein gray values was used for the relative content of this protein.

Biochemical tests

The whole tendon complex was removed to determine the extent of its function by a mechanical test. The whole Achilles tendon was removed from the heel bone to the junction of muscle and tendon and fixed to the mechanical tester (Bang Instrument Co., LTD., Suzhou, China). The tendon was firmly held, and the tendon healing place placed in the center of the fixture. A 1 N power was loaded and set prior to testing. The tendon was maintained with the appropriate tension, and the initial length was measured. The tendon was first stretched 0.5 mm at the speed of 0.5 mm/s three times for the pre-processing. The tendon was continuously loaded at a speed of 1 cm/min until the tendon ruptured. The maximum tensile strength and gliding distance at the point of reaching the maximum tensile strength of the tendon were recorded for data processing.

Hematoxylin-eosin (HE) staining

An automatic embedding machine LEICA EG 1160 was used for paraffin embedding and a LEICA RM 2235 rotary microtome was used to slice the tissues into a thickness of 4 μ m. The tissue slices were heated at 60°C until the wax dissolved; then, they were immersed in xylene twice for 5 min each time. Tissue slices were then immersed in 100% alcohol twice, 95% alcohol twice, 90% alcohol twice, 80% alcohol twice, and

washed with running water twice, one minute each time. Slices were immersed in a Harris hematoxylin liquid for 10 minutes before being flushed with tap water for 1 min, immersed in 1% hydrochloric acid alcohol for 30 s for differentiation, and flushed with running water for 15 min to turn the slices blue. Next, the slices were immersed in 1% eosin alcohol for 3 min for coloration, in 90% alcohol for 30 s for differentiation, in 95% alcohol for 1 min for washing, and in dimethyl carbonate for 1 min for washing before washing three times with dimethylbenzene, each time for 2 min. Finally, neutral gum (GT21316; Huayueyang Biotechnology (Beijing) CO., LTD., Beijing, China) was used as a sealant. The morphological structure of the rats' tendons before thermal preconditioning and 4 weeks after surgery were observed under a light microscope. Histological characteristics of tendon healing were observed under a light microscope. Using Image-Pro Plus 5.0 software, each HE stained section was evaluated at 100x microscopy for fiber analysis.

Masson staining

We performed the same procedures of fixation, dehydration, embedding, sectioning, and dewaxing of slices were the same as for HE staining. The slices were first dewaxed with distilled water and stained with Weigert hematoxylin for 5–10 minutes. The slices were washed with water, differentiated with hydrochloric alcohol, and rinsed with running water for several minutes. The slices were then stained with acid fuchsin solution for 5 minutes, washed with distilled water, and treated with 1% potassium permanganate solution for 5 minutes. Under the microscope, the muscle fibers appeared red and collagen fibers were light red. The slices were directly stained with aniline blue or bright green liquid for 3–5 minutes, and treated with 1% glacial acetic acid for 1 minute. Afterward, they were treated with 95% alcohol for anhydrous alcohol dehydration, xylene transparent, and neutral gum sealing. Under the microscope, the collagen fibers appeared blue (with aniline blue) or green (with bright green liquid); the muscle fibers, cytoplasm and red blood were stained red and the cell nuclei was seen as blueish brown. Each step was used to control the dyeing effect under the light microscope. The changes in the collagen fiber content were observed with Masson staining.

Immunohistochemical staining

The tissue slices were conventional fixed with water and then treated with 3% hydrogen peroxide for 1 hour to block endogenous peroxidase activity. PBS solution was used to wash the slices three times, 2 minutes each time. Anti-β-catenin (1:1000, ab16051), EPCAM (1:100, ab71946), FGF-2 (1:500, ab8899), FGF-1 (1:500, ab63601), Tenomodulin (1:500, ab203676) or scleraxis (1:1000, ab58655) were added to the tissue slices and incubated overnight at 4°C. All antibodies used were purchased from Abcam Inc., (Cambridge, MA, USA). The slices were removed and washed with PBS solution three times, 2 minutes each time. The HRP-labeled sheep anti-rabbit secondary antibody (1:500, ab6721, Abcam Inc., Cambridge, MA, USA) was added to the slices and incubated for 30 min at room temperature, followed by three times rinse with PBS solution. 3,3'-diaminobenzidine (DAB) (ZLI-9034; Beijing Zhongshan Golden Bridge Biotechnology Co., LTD., China) was used to colour the slices, until we observe a brown precipitate appeared under the microscope. The coloration was terminated with running water, which was followed by redyeing with hematoxylin in the nucleus, bluing with water, anhydrous alcohol dehydration and sealing with neutral gum. Three visual fields of each sample were randomly selected for observation at 10 × magnifications. Image-Pro Plus 6.0 software was used to measure the semi-quantitative value of the mean light density of positive cells (tan particles in the cells were considered positive). Total number of cells and number of positive cells were calculated. The ratio of the positive cell rate equals the number of positive cells divided by the number of total cells, which gave us the relative expression of the target protein in the cell.

Statistical analysis

SPSS 19.0 software was used for data analysis. Data collected were expressed as a mean ± standard deviation (SD) and tested for normality. The comparisons for significance within the measurement data were conducted using the *t* test. The χ^2 test was used for comparisons within the enumeration data. Spearman correlation analysis was applied for correlation analysis. The receiver operating characteristic curve (ROC curve) was used to analyze the diagnostic value of HSP72 mRNA and protein expression levels in tendon adhesion at the 8th week after surgery. Value of *P* < 0.05 was considered statistically significant.

Results

Tendon adhesion score comparison between the thermal preconditioning and control groups

During the 4th week after surgery, both the thermal preconditioning and control groups showed tendon adhesion, weak gliding function and significant adhesion to surrounding tissues. The tendon adhesion scores in the control group were calculated to be 4.07 ± 0.46 and 4.00 ± 0.38 in the thermal preconditioning group; there was no significant difference between the both groups ($P > 0.05$). However, during the 8th week after surgery, the tendon adhesion lessened in both groups; however, compared to the control group, the tendon adhesion in the thermal preconditioning group was much less whereby most of the adhesions could easily be separated by blunt dissection ($P < 0.05$). The tendon adhesion scores of the control group was significantly higher than the thermal preconditioning group with a score of 2.80 ± 0.38 versus a score of 1.27 ± 0.46 ($P < 0.05$) (Fig. 1).

mRNA and protein expression levels of HSP72 between the thermal preconditioning and control groups

No difference in the mRNA or protein expression levels of HSP72 could be observed in the thermal preconditioning and control groups after the 4th week of surgery (both $P > 0.05$). However, during the 8th week after surgery, elevated expressions of mRNA and HSP72 could be detected compared to those measured at the 4th week after surgery (both $P < 0.05$). Additionally, the mRNA HSP72 expressions in the thermal preconditioning group were significantly higher than in the control group at the 8th week after surgery ($P < 0.05$). At the 8th week after surgery, mRNA expression of HSP72 in the thermal preconditioning group was approximately 1.4 times higher than that of the control group (Fig. 2A), while the protein expression of HSP72 in the thermal preconditioning group was approximately 1.6 times higher than the control group (Fig. 2C). Consistent with Fig. 1, the ROC curve analysis of the HSP72 mRNA and protein expression levels in tendon adhesion at the 8th week after surgery, the cutoff points for the mRNA and protein expression levels of HSP72 were 2.05 and 1.98. Additionally, whereas their sensitivity and specificity scores were 0.73 and 1.00 and their specificity was 0.87 and 1.00, respectively (Fig. 2D~E).

The maximum tensile strength and gliding distance of tendons between the thermal preconditioning and control groups

Biomechanical testing of the two groups of rats at the 8th week after surgery showed no significant difference in the tensile strength between the two groups ($P > 0.05$). Although the broken ends of the tendons in the control group healed, the gliding distance in the control group was significantly shorter than that in the thermal preconditioning group due to severe tendon adhesion within the surrounding tissues and poor tendon recovery ($P < 0.05$) (Table

Fig. 1 Comparison of tendon adhesion at the 4th and 8th week after surgery between the thermal preconditioning and control groups (n = 15). Note: *, $P < 0.05$, compared with the 4th week after surgery and #, $P < 0.05$, compared with the control group at the 8th week after surgery.

Fig. 2. Comparison of mRNA and protein expression levels of HSP72 between the thermal preconditioning and control groups (n = 15). Note: (A) Relative expression of HSP72 mRNA with the mRNA expression in the control group before thermal preconditioning is considered as 1; (B) Western blotting showing of HSP72 protein expression in the two groups; (C) HSP72 protein expression; D, ROC curve analyzing the diagnostic value of HSP72 mRNA expression in tendon adhesion at the 8th week after surgery; E, ROC curve analyzing the diagnostic value of HSP72 protein expression in tendon adhesion at the 8th week after surgery; *, $P < 0.05$, compared with the 4th week after surgery; #, $P < 0.05$, compared with the control group at the 8th week after surgery.

Table 2. Comparison of the maximum tensile strength and sliding distance for tendons between the thermal preconditioning and control groups

	Control group (n = 15)	Thermal precondition group (n = 15)	<i>p</i>
Maximum tensile strength (N)	84.05 ± 6.34	85.23 ± 7.40	0.510
Sliding distance (mm)	2.12 ± 0.27	3.45 ± 0.40	< 0.001

Differences in morphological structure of the tendon between the thermal preconditioning and control groups

4 weeks after surgery, collagen fibers in the tendon anastomosis in the two groups were disordered and irregularly arranged. There were no differences in the shape and structure between the two groups. In both groups, it can be seen that the structure of collagen fiber was better arranged 8 weeks after surgery compared to that 4 weeks after surgery. After the 8 weeks, the scar tissue in the cutting end of the Achilles tendon in the thermal preconditioning group was more mature than the control group, and its alignment was superior to that of the control group. The results of Masson staining under a light microscope showed that after the 4th weeks following surgery, the thermal preconditioning group with a wide variety of morphological changes that include proliferation of many fibroblasts, black nuclei, regularly arranged collagen fibers, obvious hyperplasia, collagen bundles of the same diameter, and green-dyed fiber. The control group had substantial fibroblast proliferation,

Fig. 3. Sagittal sections of the Achilles tendon after surgery detected by HE staining and Masson staining. Note: A, Sagittal sections of the Achilles tendon after surgery detected by HE staining and Masson staining ($\times 100$); B, statistics for two groups of postoperative fibroblast count ($n = 15$).

Fig. 4. Comparison of the expression levels of FGF-2, FGFR-1, β -catenin, Tenomodulin and EPCAM between the thermal preconditioning and control groups. Note: A, expressions of FGF-2, FGFR-1, β -catenin, Tenomodulin, scleraxis and EPCAM between the thermal preconditioning and control groups detected by immunohistochemical staining ($\times 200$); B, positive expression rates of FGF-2, FGFR-1, β -catenin, Tenomodulin, scleraxis and EPCAM between the thermal preconditioning and control groups; C, mRNA expression of FGF-2, FGFR-1, β -catenin, Tenomodulin, scleraxis and EPCAM between the thermal preconditioning and control groups detected by RT-PCR; D, protein expression of FGF-2, FGFR-1, β -catenin, Tenomodulin, scleraxis and EPCAM between the thermal preconditioning and control groups detected by Western blotting; *, $P < 0.05$ compared with the control group ($n = 15$).

disorderly collagen fibers, collagen bundles of different degrees and a red-green color. At the 8th week after surgery, the thermal preconditioning group had many regularly arranged collagen fibers, collagen bundles of the same diameter, and red-dyed mature collagen fibers, while the control group had disorderly arranged collagen fibers, collagen bundles of different diameters and a mature red-green color (Fig. 3A). Analysis of fibroblast counts in each group by Image-Pro Plus software revealed that there was a significant delay in the fibroblast proliferation in the thermal preconditioning group (Fig. 3B).

Fig. 5. Correlation of HSP72 mRNA and protein expression with tendon adhesion at the 8th week after surgery. Note: A, correlation of HSP72 mRNA expression with tendon adhesion at the 8th week after surgery; B, correlation of HSP72 protein expression with tendon adhesion at the 8th week after surgery (n = 30).

The expression levels of FGF-2, FGFR-1, β -catenin and EPCAM in the thermal preconditioning and control groups

We found that expression levels of FGF-2, FGFR-1, β -catenin, Tenomodulin and scleraxis were higher, while EPCAM expression levels were lower than in the control group ($P < 0.05$) (Fig. 4). Therefore, we concluded that FGF-2, FGFR-1, β -catenin, Tenomodulin, scleraxis and EPCAM were associated with the degree of tendon adhesion. They were high for FGF-2, FGFR-1, β -catenin, Tenomodulin and scleraxis and high for EPCAM expression, indicating severe tendon adhesion.

Correlation of HSP72 mRNA and protein expression levels with tendon adhesion

The tendon adhesion scores with HSP72 mRNA and protein expression levels for rats in the thermal preconditioning and control groups at the 8th week after surgery were analyzed by Spearman correlation analysis. Results showed that the HSP72 mRNA and protein expression levels were negatively correlated with the degree of tendon adhesion, suggesting that low HSP72 mRNA and protein expression might indicate serious tendon adhesion and a high tendon adhesion score (Fig. 5).

Discussion

Tendon stem cells (TSCs), prone to osteogenic differentiation under excessive tension, present a high multi-differentiation potential, self-renewal capacity, and low immunogenicity [18, 19], and disordered differentiation of TSCs in the repair of injured tendon might lead to the pathogenesis of chronic tendinopathy [20]. The metaplasia of adipose tissue appeared in the ruptured tendon, which demonstrates that Dex could induce TSCs to differentiate into adipocytes, while the mechanism remains unclear [21]. Our investigation of the association between HSP72 expression after thermal preconditioning and adhesion of tendons showed that thermal preconditioning might increase the HSP72 mRNA and protein expression levels and reduce tendon adhesion after tendon surgery. Thermal preconditioning is an easily applied treatment method to prevent the adhesion of tendons.

In the present study, we found that although there was no difference in mRNA and protein expression levels of HSP72 between both groups at the 4th week after surgery, we detected elevated levels of expression later on during the 8th week after surgery. Interestingly, the thermal preconditioning group presented with a significantly higher expression levels of HSP72 mRNA and protein than the control group. In the meantime, the findings indicated that the HSP72 mRNA and protein expression levels were negatively related to tendon adhesion and the tendon adhesion score. We speculate that the up-regulated expression of HSP72 was observed in the 4th week after surgery in both groups was due to the surgical procedure itself which has been previously reported to increase HSP72 in some instances [22]. Additionally, we also found that the thermal precondition group showed higher expression of HSP72 in the 8th week after the surgery than the control group, which could be supported by the statement that thermal preconditioning can activate HSP72 [23]. The production of the flexor tendon graft requires the expansion of primary cells. During tendon

damage and collagen synthesis, the cells that have multiple fibripositors and cytoplasmic protrusions can be found in the area [4]. HSP72 is the most important member of the heat-shock protein family [24]. Studies have confirmed that when body cells are subjected to stressful conditions, such as hyperpyrexia, oxidation, and mechanical damage, the increased expression of HSP72 can strengthen the tolerance of cells to damage, improve the viability of the cells, and reduce cell necrosis or apoptosis caused by stress injury [25]. In line with our results, many previous studies have proposed that increased HSP72 can promote tissue repair by strengthening to the ability of cells to resist stressful damage, increased degradation of abnormal proteins, and maintain the normal biological activity of the cells through the enhancement of the stability of the internal structures, and having a protective effect [26-28]. Millar and Murrell et al. investigated the central role of HSPs in tendon disease and reported that HSPs may ultimately affect tissue rescue mechanisms in tendon pathology [28]. Barbe et al. reported that HSP72 increases in the muscle and tendon of rats that performed a repeated high demand reaching and grasping task for 6 weeks [26]. Similarly to our study, Healy et al. have also reported that thermal preconditioning of the limb before flexor tendon repair decreases inflammation and adhesion formation in a rabbit model, suggesting that it has the potential to improve the clinical outcome of flexor tendon surgery [29]. Thermal preconditioning is also proved to reduce inflammation by inducing the formation of cytoprotective HSPs [30].

Our study showed that at the 8th week after surgery, the thermal preconditioning group had a longer gliding distance and higher expression levels of FGF-2, FGFR-1 and β -catenin with lower EPCAM expression than the control group. Growth factors, such as platelet-derived growth factor-BB (PDGF-BB), insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF), are known to promote tendon healing and tendon cell proliferation [31]. FGF-2 has been reported to accelerate the initial tendon-to-bone remodeling and FGFR-1 plays a multifunctional role in bone formation and bone remodeling. β -catenin widely exists in fibroblasts and osteoblasts, and it plays an important role in regulating the proliferation, differentiation and apoptosis of these cells [32]. The early growth response 1 (EGR1) plays a vital role in the process of tendon-to-bone healing, as well as repair through the BMP12/Smad1/5/8 signaling pathway [33]. Additionally, the cyclic mechanical stretching could induce autophagy cell death in tendon-bone junctions by activating PGE2 production [34]. TOB1 can play a negative role in tendon-to-bone healing, which implying that TOB1 expression might be regulated by miR-218 [35]. The tardy healing process of tendon-to-bone junctions could be quickened through implanting tendon-derived stem cells (TDSCs) with transforming growth factor- β (TGF- β) gene silencing [36]. All growth factors, including bFGF, could increase collagen type I production, which may further reinforce the histological and biomechanical properties of regenerative tendons [37]. EPCAM expression has been found to be associated with cellular differentiation. Findings show that the germinal regions in normal colonic crypts display increased EPCAM expression but as the cells differentiate and move to the top of the villi, its expression decreases [38]. The reason for the reduction in EPCAM expression might be due to the reduction of adhesion between the tendon and surrounding tissues after thermal preconditioning.

In conclusion, our results demonstrated that thermal preconditioning could reduce tendon adhesion via up-regulating of HSP72 mRNA and protein expression levels following tendon surgery. Thermal preconditioning is an easily applied clinical treatment method to prevent tendon adhesion. As of now, the precise mechanism of how thermal preconditioning is yet to be determined. Future investigation is requiring elucidating its possible association with other molecules and pathways.

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Disclosure Statement

The authors declare no conflict of interest.

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