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## Original Article

Gait analysis combined with the expression of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB during Achilles tendon healing in ratLi-Ming Wu <sup>a</sup>, Jing-Kun Wang <sup>a</sup>, Jun Liu <sup>a</sup>, Chao-Chao Fan <sup>a</sup>, Yun-Jiao Wang <sup>b</sup>, Yan Xiong <sup>a,\*</sup><sup>a</sup> Department of Orthopaedics, Daping Hospital, Army Medical University, Chongqing, 400042, China<sup>b</sup> Department of Sports Medicine Center, State Key Laboratory of Trauma, Burn and Combined Injury, Southwest Hospital, Army Medical University, Chongqing, 400042, China

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## ABSTRACT

**Purpose:** To observe the changes of gait behavior and the expression of wound healing factors of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), TGF- $\beta$ 3 and cAMP response element binding protein-1 (CREB-1) during the healing of Achilles tendon in a rat model, and to investigate whether gait analysis can be used to evaluate the tendon healing.

**Methods:** Achilles tendon of 40 healthy male Sprague-Dawley rats were transected and sutured to establish the Achilles tendon injury (ATI) model. They were randomly divided into 4 groups based on the observational time point at 1, 2, 4 and 6 weeks after injury ( $n = 10$  for each group). Before modeling, 9 rats were randomly selected for CatWalk gait analysis, which contained step cycle, single stance time and average speed. Data were recorded as the normal controls. After then, ATI models were established in the left hind limbs of the all 40 rats (ATI group), while the right hind limbs were only cut and sutured without injury of the Achilles tendon (sham operation group). At 1, 2, 4 and 6 weeks after injury, the gait behavior of the corresponding group of rats ( $n = 9$ ) as observed and recorded by CatWalk platform. After then, the rats were sacrificed and Achilles tendon of both limbs was harvested. The tendon healing was observed by gross anatomy and histological examination, and the protein and mRNA expression of TGF- $\beta$ 1, TGF- $\beta$ 3, CREB-1 were observed by immunohistochemistry and qPCR. The results of tendon gross grading were analyzed by Wilcoxon rank sum test, and other data were analyzed by one-way analysis of variance among multiple groups.

**Results:** Compared with normal controls, all gait indexes (step cycle, single stance time and average speed) were greatly affected following ATI, which however improved with time. The step cycle was significantly lower at 1, 2 and 4 weeks after ATI (compared with normal controls, all  $p < 0.05$ ), but almost returned to the normal level at 6 weeks ( $(0.694 \pm 0.102)$  vs.  $(0.503 \pm 0.094)$  s,  $p > 0.05$ ). The single stance time of the ATI group was significantly shorter at 1 and 2 weeks after operation ( $(0.078 \pm 0.010)$  s at 1 week,  $(0.078 \pm 0.020)$  s at 2 weeks, all  $p < 0.001$ ) and revealed no significant difference at 4 weeks ( $p = 0.120$ ). The average speed of ATI group at 1, 2, 4, 6 weeks was significantly lower than that in the normal control group (all  $p < 0.001$ ).

Gross observation showed that the grade of local scar adhesion in ATI group increased significantly at 2, 4 and 6 weeks, compared with the sham operation group (all  $p < 0.001$ ). Extensive adhesion was formed at 6 weeks after ATI. The results of HE staining showed that the number of fibroblast increased gradually and arranged more orderly in ATI group at 1, 2 and 4 weeks (all  $p < 0.001$ ), and decreased at 6 weeks, but it was still significantly higher than that of the sham operation group ( $p < 0.001$ ). Immunohistochemistry showed that the positive expression of TGF- $\beta$ 1, TGF- $\beta$ 3, CREB-1 in ATI group was higher than that in the sham operation group at 4 time points (all  $p < 0.05$ ), which reached the peak at 2 weeks after operation and decreased at 4 weeks ( $p = 0.002$ ,  $p < 0.001$ ,  $p = 0.041$ , respectively). The results of qPCR suggested that the mRNA expression of TGF- $\beta$ 1, TGF- $\beta$ 3, CREB-1 in ATI group was higher than that in the sham operation group at all-time points (all  $p < 0.05$ ), which reached the peak at 2 weeks after operation, decreased at 4 weeks, and significantly decreased at 6 weeks (all  $p < 0.001$ ).

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**Conclusion:** Gait behavior indexes are associated with Achilles tendon healing. The study gives an insight of TGF- $\beta$ 1, TGF- $\beta$ 3, CREB-1 changes in the coursing of Achilles tendon healing and these cytokines may be able to be used to regulate the Achilles tendon healing.

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## Introduction

Tendon is a kind of dense connective tissue, which can transfer the strength of muscle to the bone and make the limb move. It bears great strength in human activities.<sup>1</sup> Achilles tendon is the thickest tendon tissue in human body, and it is also the most vulnerable to be injured in daily life.<sup>2,3</sup> Lemme et al.<sup>4</sup> have reported that the incidence of Achilles tendon rupture was 2.5 per 100,000 person-years in 2016 in the United States. The treatment of Achilles tendon includes conservative and surgical managements. Due to the lack of sufficient vascular tissue, the Achilles tendon is mainly repaired by scar after injury, and there is still a secondary rupture rate of 1.6% after successful repair.<sup>5</sup>

With the improvement of surgical methods in recent years, minimally invasive anastomosis, suspension fixation, arthroscopic assisted percutaneous anastomosis and other methods have been used to reduce the damage of surrounding tissues. An early post-operative functional exercise combined with physical therapy indeed reduced the occurrence of surgical complications, but the studies showed the rate of secondary rupture is still not changed significantly.<sup>5,6</sup>

The main causes of Achilles tendon secondary rupture are the lack of enough endogenous healing strength, and re-injury caused by improper postoperative exercise gait. The healing mechanism of Achilles tendon injury (ATI) includes endogenous healing and exogenous healing.<sup>7</sup> Exogenous healing mainly depends on the growth of fibrous connective tissue into Achilles tendon to form scar tissue. Endogenous healing refers to the division and proliferation of fibroblasts in Achilles tendon itself, adventitia or blood vessels during the healing process, and through the proliferation of its own cells. And then, the normal Achilles tendon collagen fibers are formed to participate in the repair process. The strength of Achilles tendon healed by normal Achilles tendon collagen fiber is greater than that healed by scar tissue. Inhibiting exogenous scar healing and enhancing endogenous intra-tendon healing can improve the final healing strength of Achilles tendon. Therefore, the mechanism regulation of Achilles tendon endogenous healing has become a hotspot research.

The endogenous healing process of Achilles tendon is regulated by a variety of cytokines. Some recent studies reported that transforming growth factors (TGF)- $\beta$ 3, TGF- $\beta$ 1 and cAMP response element binding protein-1 (CREB-1) play important roles in the healing process of tendon injury and have a certain impact on the formation of scar healing,<sup>8–11</sup> which may affect the tendon healing and scar formation through CREB/TGF- $\beta$ 3, TGF- $\beta$ /Smads signaling pathways and others. The expression of these factors in the process of Achilles tendon healing may be related to the quality of Achilles tendon healing, and then affect the secondary rupture of Achilles tendon. Although the changes of inflammatory factors have a certain predictive effect on Achilles tendon healing, the regularity of gait changes and the healing strength of Achilles tendon need to be further studied.

Therefore, this experiment using rat ATI model, observed the relationship between animal gait changes and Achilles tendon healing, and analyzed the expression of related wound healing

cytokines, in order to provide a new research idea for promoting endogenous healing of Achilles tendon.

## Methods

### Experimental materials

#### Animals

We used 40 healthy male Sprague–Dawley rats (provided by the experimental animal center of Daping Hospital of Army Medical University), weighing 180–200 g, were fed in single cages.

#### Main instruments and reagents

The main instruments and reagents used in this study included Catwalk XT (Noldus information technology, Netherlands), high-speed freezing centrifuge (Kejun instrument company, USA), Nanodrop ultraviolet spectrophotometer (thermofisher, USA), fluorescence quantitative PCR (Pall, 65421), paraffin rotary slicer (Leica, Germany), tissue RNA extraction kit (SGG b618133-0050), cDNA synthesis kit (Sangon Biotech, B532445-0020), qPCR mix (Sangon Biotech, B110031-0001), immunohistochemistry kit (Sangon Biotech, E607250-0100), anti TGF- $\beta$ 1 antibody (Abcam ab92486), anti-TGF- $\beta$ 3 antibody (Abcam ab15537), anti-CREB antibody (Abcam ab31387).

### Experimental methods

#### Animal grouping and experimental intervention

The experimental animals were randomly divided into 4 groups with 10 rats in each group. Before modeling, one group was randomly selected for gait analysis, and the data was recorded as a normal control. The expression of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 was evaluated. All subjects had established ATI models on their left hind limbs, which were recorded as ATI group. The right hind limbs were cut and sutured without injury of the Achilles tendon, defined as the sham operation group.

#### Establishment of animal model

The Sprague–Dawley rats were anesthetized by abdominal cavity. After the success anesthesia, the heel was shaved locally for skin preparation. After disinfection, the skin and subcutaneous tissue were cut posterior along the long axis, and then the flaps were retracted to both sides for exposing the Achilles tendon. The Achilles tendon of the left hind limb was transected with a blade at the midpoint of muscle tendon transition and the stop site of Achilles tendon.<sup>12</sup> The modified Kessler suture was used to repair the Achilles tendon with 5-0 absorbable suture. The Achilles tendon of the right hind limb was not transected after skin incision and there was no other intervention. The skin was sutured with 2-0 absorbable suture. The operating foot was not fixed specially and all rats were kept in single cages under the same conditions.

#### Acquisition of experimental specimens

At respectively 1, 2, 4 and 6 weeks after establishing the repair model of ATI, samples were collected from both hind limbs (ATI side

and sham operation side). An incision was made along the original surgical incision after the animals were anesthetized, the surrounding adhesion was separated, and the healing Achilles tendon was exposed and extricated. The Achilles tendon specimens within 1 cm of the distal and proximal end of the suture were quickly and completely cut off. After suturing the skin wound, normal feeding was continued.

#### Observation indexes

In this experiment, we analyzed the changes of gait behavior and observed the grade of tendon healing. Gait analysis reflects the rat behavioral indicators. Gross observation can directly find the tendon healing. HE staining reflects the healing of tendon from the histological level. CREB-1 may regulate the TGF- $\beta$  and influence the local tendon scar formation.<sup>13,14</sup> So we performed tendon HE staining, immunohistochemical test and qPCR detection of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1.

#### Behavioral observation

On the day of sampling, CatWalk platform was used to observe the animal gait, and the data collected by the platform were analyzed and processed. We mainly analyzed and compared the parameters of step cycle, single stance and average speed. The step cycle (s) is the time between 2 successive contact glass plates by the same claw, and only the data of the ATI limb were analyzed. The average speed (cm/s) is the speed through the glass plate. Both the step cycle and average speed are parameters related to mobility analysis with short step cycle and high average speed suggesting that the animal has good motor function. The single stance (s) is the time that only the affected limb touches the glass plate. The pain of the affected limb will lead to short single leg support time, and therefore the longtime of single stance indicates that the animal has good motor function.

#### Gross observation

When Achilles tendon specimens were obtained at each time point, the healing of the exposed Achilles tendon was observed by light microscope and recorded by photography. According to the healing of Achilles tendon observed, the Achilles tendon specimens were graded respectively.<sup>15</sup> Grade I: There was no adhesion around the tendon and granulation tissue could exist. Grade II: There were a few localized membranous adhesions at the tendon suture, granulation tissue existed, and tendon slip was slightly limited. Grade III: There were small bands of loose adhesions, which can be separate from tendon surface easily, and the slippage of tendon is partial limited. Grade IV: There were moderately dense adhesions, tendon has certain extent mobility and the movement was obviously limited. Grade V: There were severe extensive adhesion, poor mobility and no boundary between tendon and peritendinous tissue. The healing of Achilles tendon was compared and analyzed.

#### Histological observation

After anatomical observation at each time point, the obtained Achilles tendon tissue was fully fixed and wax-embedded, and 6  $\mu$ m paraffin section were prepared. Stained with HE, the sections were used to observe the number of fibroblast and collagen arrangement at the Achilles tendon broken ends under the microscope, of which photos were taken under the 400 times light microscope. Six good visual fields were randomly selected from each section, and the cells in each visual field were counted by Image-Pro plus 6.0 software. The number of cells was counted and the differences between groups were analyzed.

#### Immunohistochemical test

Three sections of the paraffin embedded Achilles tendon were prepared for immunohistochemical staining. Methods: dewaxing to water after baking slices, boiling to repair antigen, blocking by 3% H<sub>2</sub>O<sub>2</sub>, incubating overnight at 4 °C by antibody against TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1. The IgG of biotin labeled goat anti-rabbit was added after incubation, DAB was used to develop color, and hematoxylin was used to dye again. Under 400 times light microscope, 6 well stained visual fields were randomly selected and photographed from each section. The location and quantitative analysis of the expression of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 were carried out by photos, and the Image-Pro plus 6.0 software was used for quantitative analysis.

#### Relative quantification by qPCR

The Achilles tendon tissue samples of the hind limbs at each time point were fully ground after adding liquid nitrogen. The total RNA was extracted by the kit to determine the concentration, and the extracted RNA was reversely transcribed by the cDNA synthesis kit. The DNA primer of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 were searched according to the GenBank, and  $\beta$ -actin was the internal reference primer. SYBR method was used for PCR reaction. The relative quantitative method was used to determine the mRNA expression of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 in ATI group and the sham operation group at each time point, and the results were compared and analyzed.

#### Statistical analysis

SPSS 25.0 statistical software was adopted. Wilcoxon rank sum test was used to analyze the grade of Achilles tendon healing under microscope, and the analysis of variance was used to test the data among multiple groups. The data of behavior, HE staining, immunohistochemistry and qPCR were displayed as the mean  $\pm$  standard deviation. Analysis of variance was used for comparisons among multiple groups, and *t*-test was used for comparisons between two groups. *p* < 0.05 was considered to be statistically significant.

## Results

#### Gait behavior

Three indexes of step cycle, single stance time and average speed were recorded by CatWalk platform before sampling at each time point, for gait analysis of the left hind limbs after ATI. Compared with normal controls (0.503  $\pm$  0.094) s, the step cycle was longer after ATI at all-time points, more specifically significantly longer at 1 (1.055  $\pm$  0.259) s, 2 (0.973  $\pm$  0.221) s and 4 (0.732  $\pm$  0.330) s weeks after injury (all *p* < 0.05). At 6 weeks (0.694  $\pm$  0.102) s, the step cycle gradually returned without significant difference between normal controls (*p* = 0.074). Comparison of the step cycle between each time point only revealed significant difference between 2 weeks and 4 weeks after injury (*p* = 0.026).

The single stance time showed similar trend of step cycle, which was shorter than the normal controls at each time point. But the single stance time improved quicker. When compared with normal controls (0.124  $\pm$  0.022) s, it only show significant difference at 1 weeks (0.078  $\pm$  0.010) s and 2 weeks (0.078  $\pm$  0.020) s, but no significant difference at 4 weeks (0.110  $\pm$  0.027) s and 6 weeks (0.113  $\pm$  0.011) s. Data at 2 weeks and 4 weeks revealed great difference (*p* = 0.001).

Different from step cycle and single stance time, the average speed was significantly slower than normal controls (28.693  $\pm$  3.350) cm/s at all-time points (9.207  $\pm$  3.159) cm/s at 1

week, ( $10.324 \pm 3.023$ ) cm/s at 2 weeks, ( $17.194 \pm 4.583$ ) cm/s at 4 weeks and ( $20.393 \pm 3.747$ ) cm/s at 6 weeks, (all  $p < 0.001$ ). The average speed at 1 week was the slowest and gradually increased in the next time. Data at 2 weeks and 4 weeks also showed significant difference ( $p < 0.001$ ) (Fig. 1).

General healing

Bilateral Achilles tendons were sampled at each time point. The Achilles tendon of the left hind limb was the ATI group, and the

right side was the sham operation group. Ten rats with 20 Achilles tendons were obtained at each time point. The healing of Achilles tendon in both groups was graded.

In the sham operation group, there was no obvious adhesion between Achilles tendon and surrounding tissue, which was easy to separate. The surface of Achilles tendon was smooth, and there was no obvious proliferation of granulation tissue. In ATI group, the adhesion around Achilles tendon was gradually aggravated over time, so as the difficulty to separate Achilles tendon. The surface smoothness and sliding of Achilles tendon also decreased, and

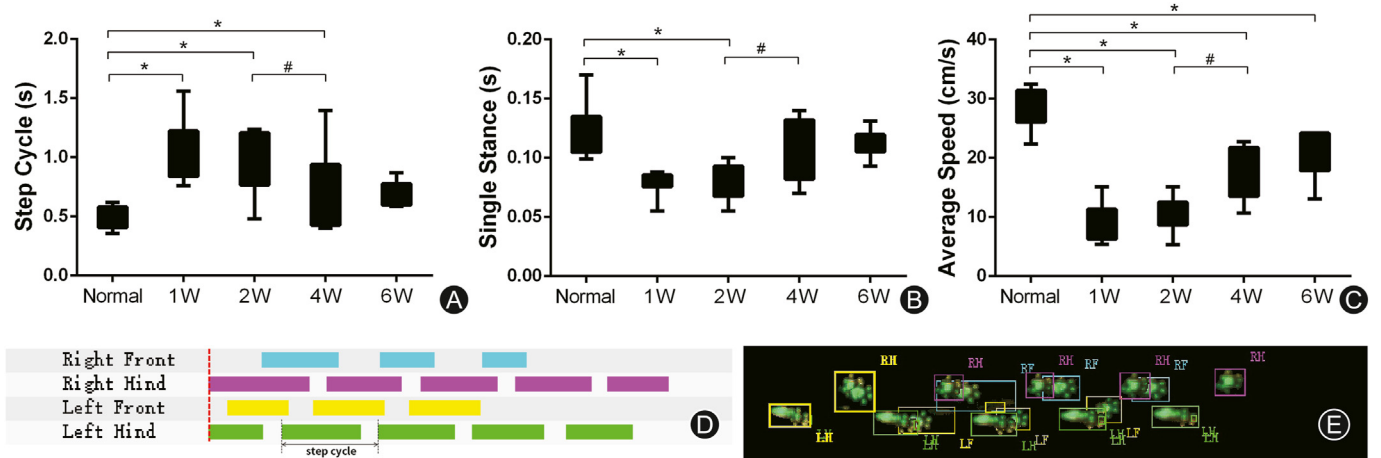


Fig. 1. CatWalk gait analysis results: (A) step cycle results, (B) single stance time, (C) the average speed, (D & E) pattern diagram of gait analysis. Normal: the control group. Data were analyzed by  $\bar{x} \pm s$  ( $n = 9$ ). \*, #: significant difference,  $p < 0.05$ .

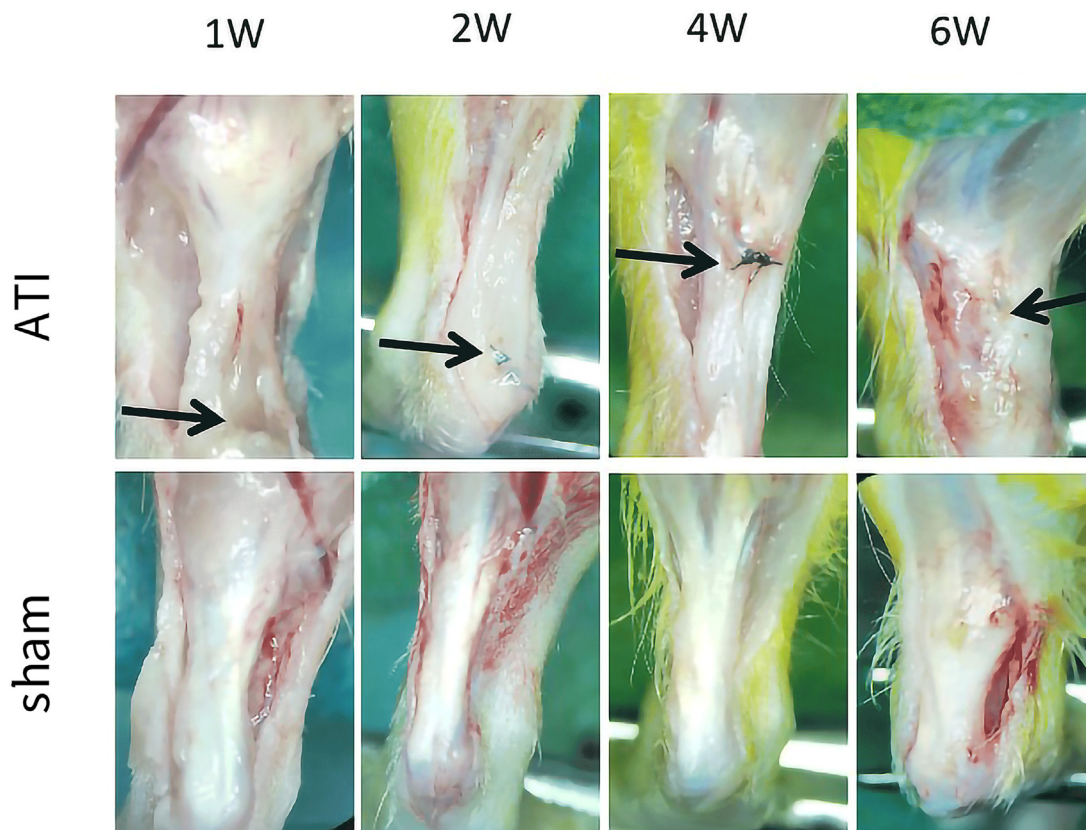
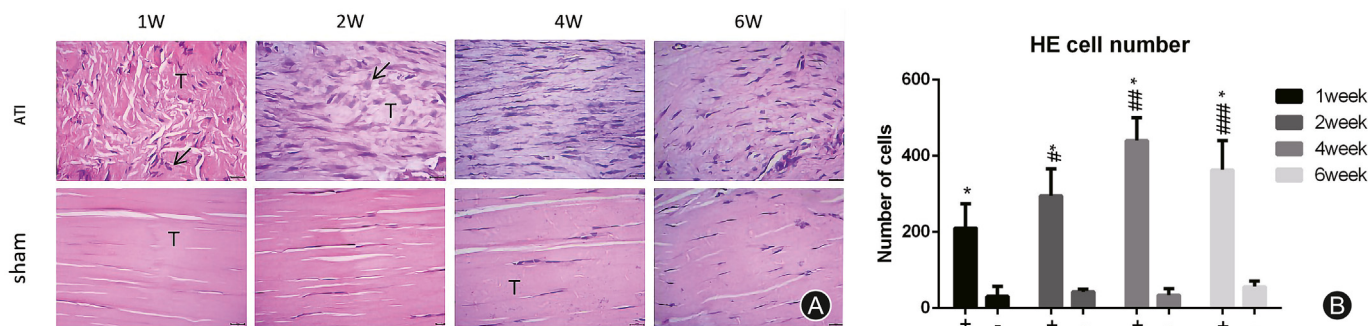


Fig. 2. The general situation of adhesion around Achilles tendon in ATI group and sham operation group at 1, 2, 4 and 6 weeks.

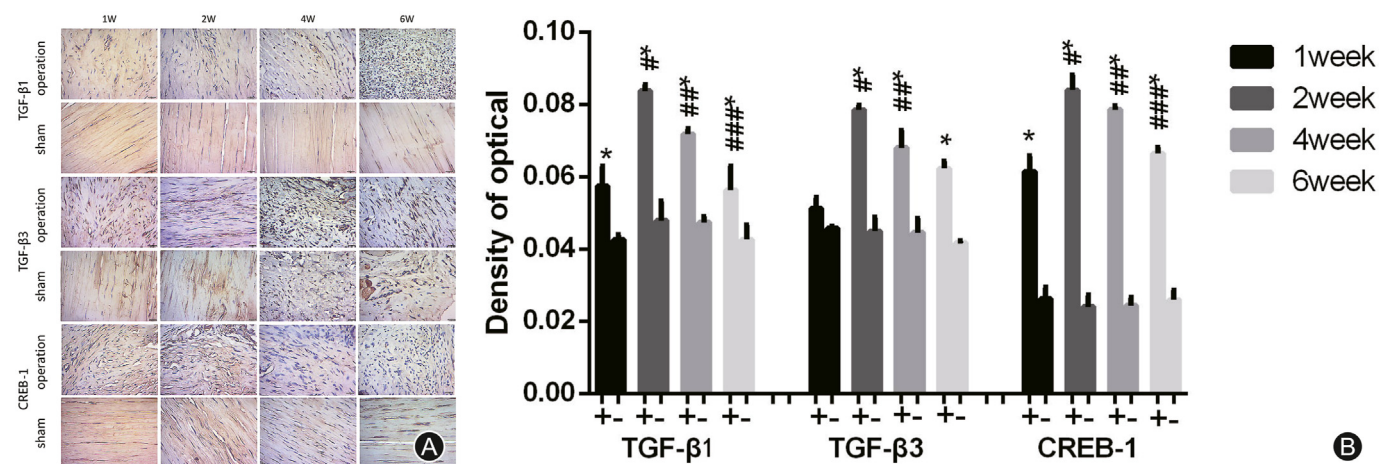


**Fig. 3.** HE staining and cell count result at each time point. (A) HE staining microscope observation of tissue sections of ATI group and sham operation group at each time point (400 × ), scale bars: 20 μm, black arrow as fibroblasts, T as collagen fibers. (B) HE staining section cell count at each time point.

+: ATI group, -: the sham operation group.

Data were analyzed by  $\bar{x} \pm s$  (n = 10).

\*: compared with the sham operation group,  $p < 0.05$ , #: compared with ATI group at 1 week,  $p < 0.05$ , ##: compared with ATI group at 2 weeks,  $p < 0.05$ , ###: compared with ATI group at 4 weeks,  $p < 0.05$ .



**Fig. 4.** The immunohistochemical results and quantitation of TGF-β1, TGF-β3 and CREB-1 at each time point. (A) Immunohistochemical microscopic observation of tissue sections of ATI group and sham operation group at each time point (400 × ), scale bars: 20 μm. (B) The immunohistochemical quantitative situation of TGF-β1, TGF-β3 and CREB-1 at each time points.

+: ATI group, -: the sham operation group.

Data were analyzed by  $\bar{x} \pm s$ , n = 10.

\*: compared with the sham operation group,  $p < 0.05$ , #: compared with ATI group at 1 week,  $p < 0.05$ , ##: compared with ATI group at 2 weeks,  $p < 0.05$ , ###: compared with ATI group at 4 weeks,  $p < 0.05$ .

extensive adhesion was formed at 6 weeks after surgery (Fig. 2). At each time point, the degree of adhesion of Achilles tendon was Graded I in the sham operation group. For ATI group, all the 10 pieces of Achilles tendon were of Grade I at 1 week, 4 pieces of Grade II and 6 pieces of Grade III at 2 weeks, all 10 pieces of Grade IV at 4 weeks, and all 10 pieces of Grade V at 6 weeks. Statistical results showed that the adhesion of Achilles tendon in the ATI group was more serious than that in the sham operation group at 2, 4 and 6 weeks (all  $p < 0.05$ ). The grade of scar adhesion was gradually increased until 6 weeks after surgery, of which the difference at the adjacent time point in ATI groups was statistically significant (all  $p < 0.001$ ).

### HE staining

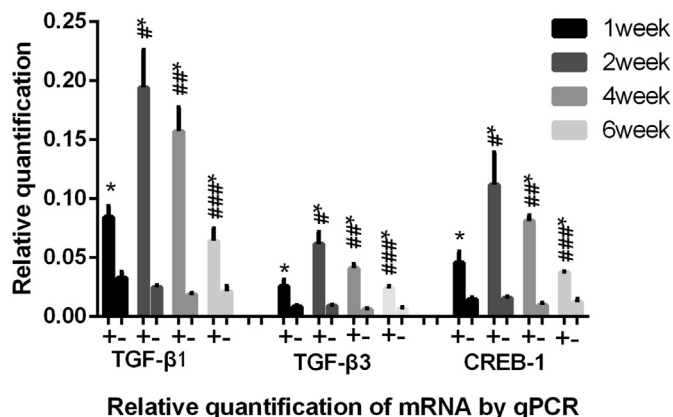
The number of fibroblast in ATI group was significantly higher than that in the sham operation group at each time point (all  $p < 0.001$ ). The number of fibroblast in ATI group increased significantly at 2 and 4 weeks ( $p < 0.001$ ), which reached the peak at 4 weeks  $440.10 \pm 57.66$ , and there was a significant decrease at 6

weeks  $363.30 \pm 66.46$ . The difference was statistically significant ( $p < 0.001$ , Fig. 3). The collagen area was small and disordered at 1 week, increased at 2 weeks, and gradually regularly arranged at 4 and 6 weeks.

### Immunohistochemical test

Immunohistochemistry showed that the positive expression of TGF-β1, TGF-β3, CREB-1 in ATI group was higher than that in the sham operation group at 4 time points (all  $p < 0.05$ ), which reached the peak at 2 weeks after operation and decreased at 4 weeks ( $p = 0.002$ ,  $p < 0.001$ ,  $p = 0.041$ , respectively).

Immunohistochemistry of Achilles tendon specimens obtained from each group showing the chain distribution of TGF-β1 and TGF-β3, and CREB-1 were scattered around the nucleus. Image-Pro software was used for quantitative analysis, and the results showed that the average optical density level of TGF-β1 in ATI group was higher than that in the sham operation group at each time point (all  $p < 0.05$ ). The average optical density level of TGF-β1 in ATI group reached the highest level at 2 weeks ( $0.084 \pm 0.002$ )



**Fig. 5.** The relative quantification results of TGF-β1, TGF-β3 and CREB-1 by qPCR at each time point.

+: ATI group, -: the sham operation group.

Data were analyzed by  $\bar{x} \pm s$  ( $n = 10$ ).

\*: compared with the sham operation group,  $p < 0.05$ , #: compared with ATI group at 1 week,  $p < 0.05$ , ##: compared with ATI group at 2 weeks,  $p < 0.05$ , ###: compared with ATI group at 4 weeks,  $p < 0.05$ .

and significantly higher than that at 1 week ( $p < 0.001$ ). Compared with that at 2 weeks, the optical density decreased significantly at 4 weeks ( $0.072 \pm 0.001$ ,  $p = 0.002$ ), and it continued to decrease significantly at 6 weeks ( $0.056 \pm 0.007$ ,  $p < 0.001$ ), but it was still significantly higher than that in the sham operation group ( $0.043 \pm 0.004$ ,  $p = 0.001$ ). In ATI group, the average optical density level of TGF-β3 at 2 weeks ( $0.079 \pm 0.001$ ), 4 weeks ( $0.068 \pm 0.005$ ) and 6 weeks ( $0.062 \pm 0.002$ ) was significantly higher than that in the sham operation group (all  $p < 0.05$ ). The average optical density level of TGF-β3 in ATI group reached highest at 2 weeks and significant higher than that at 1 week ( $p < 0.001$ ). Compared with that at 2 weeks, the level decreased significantly at 4 weeks ( $p < 0.001$ ). The average optical density of CREB-1 in ATI group at each time point was higher than that in the sham operation group (all  $p < 0.001$ ), which reached the highest at 2 weeks ( $0.084 \pm 0.004$ ), and compared with at 1 week, there was a significant increase ( $p < 0.001$ ). Compared with at 2 weeks, the level decreased significantly at 4 weeks ( $0.079 \pm 0.001$ ,  $p < 0.05$ ), and the decrease level was still significant at 6 weeks ( $p < 0.001$ ) (Fig. 4).

#### Relative expression results of mRNA by qPCR

The results of qPCR showed that was higher than that in the sham operation group at all-time points ( $p < 0.05$ ), and reached the highest at 2 weeks. In ATI group at 2 weeks, the relative expression level of TGF-1 mRNA was  $0.194 \pm 0.032$ , TGF-3 mRNA was  $0.062 \pm 0.010$ , and CREB-1 mRNA was  $0.112 \pm 0.027$ . In addition, compared with at 2 weeks group, the mRNA expression of TGF-β1, TGF-β3 and CREB-1 in ATI group showed decrease significantly at 4 weeks (all  $p < 0.05$ ), and continued to decrease significantly at 6 weeks (all  $p < 0.05$ ), but it was still significantly higher than that in the sham operation group ( $p < 0.05$ , Fig. 5).

#### Discussion

The endogenous healing is an ideal way of Achilles tendon healing. In the process of Achilles tendon healing, enhancing endogenous healing and reducing exogenous healing can improve the healing strength of Achilles tendon. Although inhibiting the exogenous healing before complete Achilles tendon healing will increase the incidence of secondary rupture, the risk can be avoided

by using deck, brace and other protective measures during this period. At present, the methods to observe and evaluate the healing of Achilles tendon include ultrasound, nuclear magnetic resonance and behavioral indicators such as single-legged concentric heel raise ability, symmetry of calf girth and ankle range of motion. But, there is no standardized process for the rehabilitation evaluation of ATI.<sup>16</sup>

In order to find a convenient and noninvasive method to dynamically observe the healing of ATI used in basic experimental research, and realize intuitive and noninvasive behavioral observation and evaluation of experimental animals, we introduced CatWalk animal gait analysis technology. There are some commonly used behavior analysis methods, such as field test<sup>17</sup> and rotation test,<sup>18</sup> which can only collect dynamic or static data of a single measurement gait, and the process is complex. However, CatWalk computer aided gait analysis system provides a systematic, convenient and automatic evaluation method, which can collect a large number of static and dynamic data at the same time<sup>19</sup> and make the behavioral evaluation more objective and comprehensive. This experiment evaluated the healing of Achilles tendon by the adhesion around the scar. The grade of adhesion around the scar was gradually increased, and extensive adhesion was formed at 6 weeks after surgery, which reflected that the scar was gradually stable and the healing of Achilles tendon was gradually improved. The results of gait analysis showed that the step cycle in ATI group gradually shortened, and the single stance time and average speed stride gradually increased, which showed that with the healing of Achilles tendon the gait behavioral indicators gradually improved. And there was no significant difference between ATI group and the normal control group regarding the step cycle and single stance time at 6 weeks after surgery, further showing that there was a certain correlation between gait and Achilles tendon healing.

TGF-β is a multifunctional protein, which can participate in the regulation of cell proliferation, differentiation and apoptosis in a variety of cells. The study found that TGF-β can increase the transcription of collagen gene in cells, and thus replace the fibrotic scar of extracellular matrix, which indicated that TGF-β is closely related to the endogenous healing of ATI. And this study reported the TGF-β1 is a key factor of scar formation and tendon adhesion in the process of tendon healing.<sup>20</sup> A study have shown that up-regulation of mRNA transcription of TGF-β1 leads to excessive scar formation,<sup>21</sup> which indicates that the regulation of the expression of TGF-β1 will contribute to regulating the scar formation. And another study have also found that TGF-β1 antibody can reduce scar adhesion.<sup>22</sup> As an isomer of TGF-β1, TGF-β3 is also a natural antagonist of TGF-β1, which is thought to counteract the scarring effect of TGF-β1.

In the last century, it has been found that pregnant fetus can achieve perfect wound healing without scarring.<sup>23</sup> The low expression of TGF-β1 and high expression of TGF-β3 were observed in the scarless healing process of fetus, which is opposite to the scar formation in adults,<sup>24,25</sup> and some studies have found that the low expression of TGF-β receptors may also contribute to scarless wound healing.<sup>26</sup> These studies have shown that TGF-β1/3 plays an important role in the formation of tendon scar. The signal of TGF-β is mainly transmitted through the downstream protein of Smad. TGF-β1 binds to the TGF-βRII receptor on the cell membrane, phosphorylates the receptor I and activates it. And then phosphorylates Smad3 in cytoplasm form a complex with Smad4 and translocate into the nucleus, regulates the transcription of the corresponding genes and promoting fibrosis. While TGF-β3 can promote the expression of Smad7 protein, which can inhibit TGF-β1 combining with receptor, degrade the TGF-β receptor by ubiquitination, inhibit the phosphorylation of Smad3, and inhibit the

formation of Smad3/4 complex and translocation to the nucleus.<sup>27</sup> TGF- $\beta$ 3 plays an anti-fibrosis role through negative feedback effect.<sup>13</sup>

CREB is a nuclear protein that can selectively bind to cAMP response element (CRE) and specifically bind to CRE. It participates in the transcriptional regulation of a variety of target genes induced by cAMP, which is also called transcriptional enhancer. Studies have found that CREB can regulate the metabolism and growth of cells,<sup>28</sup> and also participate in the regulation of tissue fibrosis and scar formation.<sup>13,29</sup> Fibrosis can occur in a variety of organs. The process of tendon healing is similar to the formation of fibrosis. It is also a fibrosis process in which the abnormal expression of a variety of cells, cytokines and nuclear transcription proteins leads to the accumulation of extracellular matrix and the formation of collagen. In the process of tendon healing, studies have found that increasing CREB phosphorylation can improve tendon healing.<sup>14</sup> These studies suggest that CREB may be a key protein regulating tendon healing. Therefore, it is particularly important to study the regulatory role of CREB-1 in Achilles tendon healing, to deeply understand the mechanism of Achilles tendon healing, and to promote the Achilles tendon healing.

The study of hepatic stellate cells, intestinal epithelial cells and rats have shown that CREB-1 is a key protein that binds TGF- $\beta$ 3 promoter to regulate TGF- $\beta$ 3 protein expression.<sup>14,30,31</sup> It is well known that the overexpressed Smad7 is able to inhibit fibrosis in various tissues via antagonizing the TGF- $\beta$ 1/Smad3 signaling pathway.<sup>32,33</sup> In hepatic stellate cells research, inhibiting the expression of CREB-1 can reduce the TGF- $\beta$ 3-induced up-regulation of Smad7, and the overexpression of CREB-1 can induce the TGF- $\beta$ 3-induced up-regulation of Smad7.<sup>13</sup> In rat intestinal epithelial cells, exogenous TGF- $\beta$ 3 can promote the increase of TGF- $\beta$ 3 transcriptional expression, leading to the increase of TGF- $\beta$ 3 secretion. The CREB-1, as the critical activators of TGF- $\beta$ 3, plays an important role in this process. Inhibiting the expression of CREB-1 can significantly inhibit the TGF- $\beta$ 3 promoter activity, TGF- $\beta$ 3 mRNA expression and TGF- $\beta$ 3 secretion.<sup>30</sup> Another study has also showed that the increase of phosphorylation of CREB-1 can inhibit the TGF- $\beta$ 1/Smad signaling pathway, and reduce the development of TGF- $\beta$ 1-mediated liver fibrosis.<sup>34</sup> And the study showed that the increase of the phosphorylation of CREB can inhibit the inflammatory response of Achilles tendon tissue during Achilles tendon healing.<sup>14</sup> All of these studies suggest that CREB-1 may influence the formation of tendon scarring by affecting the expression of TGF- $\beta$ 1/3.

In this experiment, we observed that the protein expressions of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 were at high levels during the period of extensive adhesion formation. In the scar formation process, the protein expression levels of TGF- $\beta$ 1 and CREB-1 have been significantly increased at 1 week after surgery, and the protein expression levels of TGF- $\beta$ 3, TGF- $\beta$ 1 and CREB-1 reached the peak at 2 weeks and then gradually decreased. But they were still significantly higher than that in the sham operation group at 6 weeks, indicating that the long-term maintenance of high protein expression levels of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 may be closely related to the formation of tendon scar. It is suggested that regulating the protein levels of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 in the early stage of healing may help regulate the formation of scar. With the gradual healing of tendon, the average speed and single stance of gait increase gradually and step cycle declines gradually. Although it may take more than 6 weeks for the average speed to reach a near normal level, the single stance was closed to the normal level at 4 weeks, and the single stance and step cycle are not significantly different from the control group at 6 weeks after surgery. The protein expression level of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 did not approach the normal level until 6 weeks. It shows that the recovery of gait is earlier than that of protein expression in the process of Achilles tendon healing.

In this study, we used a variety of research methods to observe the relationship between cytokines and Achilles tendon healing. However, we only used immunohistochemical method and qPCR method to quantify the protein expression, which are not full quantitative analysis methods for cytokines. In the further study, Western-blot method can be used for quantitative analysis of related proteins, which will further enhance the reliability of research evidence.

There is a certain correlation between gait and Achilles tendon healing after ATI. In the process of tendon repair, early adjustment of the levels of TGF- $\beta$ 1, TGF- $\beta$ 3 and CREB-1 may regulate the formation of tendon scar and promote the outcome of tendon healing. However, how TGF- $\beta$ 1/3 interacts with CREB-1 and the specific mechanism of feedback regulation is still unclear, and the specific correlation coefficient between gait analysis and Achilles tendon healing needs to be further studied. We will conduct further research in the following work to provide new research ideas for promoting better tendon healing.

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## Ethical statement

This study was approved by the Laboratory Animal Welfare and Ethics Committee of Third Military University (AMUWEC20201275).

## Declaration of competing interest

The authors do not have any conflict of interests.

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