Appl Biochem Biotechnol (2011) 165:1092–1106
DOI 10.1007/s12010-011-9327-7

Proteomic Analysis of Differential Protein Expression of Achilles Tendon in a Rabbit Model by Two-Dimensional Polyacrylamide Gel Electrophoresis at 21 Days Postoperation

Jiasharete Jielile · Ainuer Jialili · Gulnur Sabirhazi · Nuerai Shawutali · Darebai Redati · Jiangtao Chen · Bin Tang · Jingping Bai · Kayrat Aldyarhan

Received: 6 December 2010 / Accepted: 8 July 2011 /
Published online: 29 July 2011

Abstract Postoperative early kinesitherapy has been advocated as an optimal method for treating Achilles tendon rupture. However, an insight into the rationale of how early kinesitherapy contributes to healing of Achilles tendon remains to be achieved, and research in the area of proteomic analysis of Achilles tendon has so far been lacking. Forty-two rabbits were randomized into control group, immobilization group, and early motion group, and received postoperative cast immobilization and early motion treatments. Achilles tendon samples were prepared 21 days following microsurgery, and the proteins were separated with two-dimensional polyacrylamide gel electrophoresis. Differentially expressed proteins were first recognized by PDQuest software, and then identified using peptide mass fingerprinting, tandem mass spectrometry, and database searching. A total of

Ainuer Jialili contributed equally as the first author.

J. Jielile · A. Jialili · N. Shawutali · J. Chen
Department of Microsurgery Reconstruction, Institute of Orthopedics,
The First Teaching Hospital of Xinjiang Medical University, Urumqi, 830054, China

A. Jialili (✉)
The Sports Medicine research Center of Orthopedics Research Institute of Uyghur Autonomous Region of Xinjiang, Urumqi 830011, China
e-mail: Jingping-Bai@163.com

G. Sabirhazi
Xinjiang Institute of Ecological and Geography, The Chinese Academy of Sciences, Urumqi 830011, China

D. Redati · B. Tang · J. Bai
Department of Orthopedics, The Third Teaching Hospital of Xinjiang Medical University, Urumqi 830011, China

K. Aldyarhan
Altay Research Institute of Kazakh Traditional Medicine, Altay 836500, China

463±12, 511±39, and 513±80 protein spots were successfully detected in the two-dimensional polyacrylamide gels for the Achilles tendon samples of rabbits in the control group, immobilization group, and early motion group, respectively. There were 15, 8, and 9 unique proteins in these three groups, respectively, and some differentially expressed proteins were also identified in each group. It was indicated that some of the differentially expressed proteins were involved in various metabolism pathways and may play an important role in healing of Achilles tendon rupture. Postoperative early kinesitherapy resulted in differentially expressed proteins in ruptured Achilles tendon compared with those treated with postoperative cast immobilization. These differentially expressed proteins may contribute to healing of Achilles tendon rupture through a mechanobiological mechanism due to the application of postoperative early kinesitherapy.

Keywords Achilles tendon rupture · "Pa" bone suture · Postoperative early kinesitherapy · Cast immobilization · Centrifugal stress · Mechanobiology · Two-dimensional electrophoresis

Introduction

Achilles tendon (AT) rupture has long been recognized as a condition that can result in major disruptive injury and immense pain to patients [1]. A neglected AT rupture ensues on condition that the patients have remained untreated for a period of 4 weeks following injury, and significant dysfunction would occur if the patients remain untreated [2]. The typical techniques available for the treatment of AT rupture can be classified into two categories, namely surgical method (percutaneous surgery or open surgery) and nonsurgical method (cast immobilization or functional bracing) [3]. Although there is still no consensus with regard to the best method, a few randomized prospective trials and meta-analysis have been performed, and their results suggest that open surgical treatment is the method of choice, especially for athletes and young patients with a high level of physical activity [4, 5].

Recently, postoperative functional treatment involving early motion and weight-bearing exercises has been reported to shorten the rehabilitation period and result in improved clinical outcome [6, 7]. It was demonstrated that early motion of the ankle joint was an important factor in optimizing the treatment of AT rupture, and postoperative cast immobilization was not necessarily required. The rationale behind the success of postoperative early motion was potentially due to a promotion in proliferation, transportation, and alignment of tendon cells, which resulted in an increase in the circumference of collagen fibers and an overall reconstruction of the AT [8]. It has been demonstrated that tendons are able to respond to mechanical forces by altering their structure, composition, and mechanical properties [9], but a deep insight into the mechanobiology involved in the tendon healing process has not been achieved as yet.

Recent research suggested that differential protein expression in the ruptured AT treated with different methods could contribute to the different clinical outcomes obtained [10]. Proteins, as biological macromolecules in the human body, are essential parts of organisms and participate in virtually every process within cells. It is considered that protein expression profile could provide important functional information for genomics, and can be used as a reflection of dynamic changes of the biological system [11]. However, literature survey has shown a paucity of research in terms of proteomic analysis of AT. This present study therefore aims to investigate the significance of postoperative early motion treatment in the AT healing process at 21 days through a proteomic analysis of the differential protein expression in a rabbit model of AT injury.

Materials and Methods

Experimental Design

Forty-two New Zealand white rabbits (male, same age, weight of 2.5±0.2 kg) were provided by the Animal Centre, The First Teaching Hospital of Xinjiang Medical University, China. These rabbits were randomized into three groups according to a predetermined prospective animal experiment based on random number table [12], with group A (control group), group B (immobilization group), and group C (early motion group) each containing 15 rabbits. The experimental protocol was previously approved by the local research ethics committee.

The rabbits in group A were kept intact until they were sacrificed and extraction of protein was performed at unilateral AT, whereas the rabbits in group B and group C received a tenotomy and subsequent AT microsurgery. The tenotomy was performed at a site of 1.6 cm above the tendon's insertion into the calcaneus of unilateral AT of the rabbits following antisepsis, hypnotic induction, and local anesthesia using lidocaine hydrochloride injection. The AT microsurgery was carried out based on a novel surgical technique, namely parachute-like suture method ("Pa" bone suture) to treat AT rupture. This new technique was proposed by the present authors, and the detailed procedure is available elsewhere [13]. The rabbits in group B were treated with postoperative cast immobilization, with the knee joint in flexion at 75° and the ankle joint in plantar flexion at 90°. The rabbits in group C received a simulated postoperative early motion treatment through an induction for food and water, and the centrifugal stress motion was considered to occur during the alternative movement between standing up and squatting down. The frequency of the movement was about 150±15 times per day.

The rabbits in each group were excluded from this study if either of the following items occurred: (1) death of the rabbits, (2) loosening of the plaster cast, (3) infection of the ruptured site of the AT, and (4) occurrence of gap at the ruptured site of the AT larger than 1.0 mm. Consequently, a total of 36 rabbits were accepted for further experiment, with 13, 11, and 12 rabbits remaining in group A (control group), group B (immobilization group), and group C (early motion group), respectively.

Preparation of Samples

At day 21 following the AT microsurgery, the rabbits in these three groups were sacrificed, and the tendon tissues with a size of $0.5\times0.5\times0.5$ cm^3 were cut off from the same area of the ruptured AT. The epitenon tissues attached were removed with caution, and the samples were rinsed thoroughly three times using 0.9% saline solution (at 4 °C), and then snap frozen in liquid nitrogen. All these procedures were rapidly performed in order to ensure that the difference of rehabilitation period for the AT of the rabbits was controlled within an hour.

The AT tissues of the rabbits were thawed, cut into small pieces with scissors, and further rinsed using phosphate-buffered saline to remove any impurity involved. Then, the AT tissues (weight, 50.0 mg) were crushed with a mortar, and the protein lysates were extracted employing a cocktail solution (4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (chaps), 50 mmol/L dithiothreitol, 0.1% phenylmethanesulfonyl fluoride, 7 mol/L urea, 2 mol/L thiourea) associated with a centrifuge tube operated at a speed of 16,000 rpm for 45 min at 4 °C. The protein concentration was quantified based on Bradford method using ReadyPre 2-D Cleanup Kit (Bio-Rad Laboratories, Inc., USA).

Two-Dimensional Polyacrylamide Gel Electrophoresis

The proteins were first separated through isoelectric focusing (IEF) according to their isoelectric point. Four hundred micrograms of protein sample was loaded per 18 cm immobilized (solid state) pH gradient (IPG) strip with a nonlinear pH range of 3–10 by in-gel rehydration. Note that each strip was overlaid with 2–3 ml of mineral oil to prevent evaporation during the rehydration process. The IPG strip was then placed on the tray, and the IEF was performed at 20 °C using IPG-Phor isoelectric system (Amersham Pharmacia Biotech Inc., Sweden). The initial voltage was set at 250 V and raised stepwise until 4,000 V to remove salt. The proteins were focused for 8 h at 8,000 V.

After focusing, the IPG strip was removed and the mineral oil was drained off the IPG strip using wet filter paper. Proteins immobilized on the IPG strip were then placed on 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) (thickness, 1 mm) and separated based on their molecular weight using Protean II electrophoresis system (Bio-Rad Laboratories, Inc., USA) at 15 mA/gel. Subsequently, the two-dimensional gel was silver stained with reference to a modified method which enabled a direct mass spectrometry characterization [14].

The gel was then scanned using a GS-800 molecular imaging system (Bio-Rad Laboratories, Inc., USA), and spot detection, spot matching, and quantitative intensity analysis were performed using PDQuest software version 7.0. The gel images were normalized according to the total quantity in the analysis set, and differentially expressed proteins were defined through measuring the density values of the protein spots. A difference in the abundance of the protein spots equal to fivefold was nominated as the threshold. This was analyzed employing Student's *t* test based on the six density values obtained for one protein spot, and a probability value of <0.05 was considered statistically significant.

Mass Spectrometry Analysis

Differentially expressed spots were excised from the gel using a spot cutter (Amersham Pharmacia Biotech Inc., Sweden), and proteins were in-gel digested with trypsin and extracted as peptides based on the method described by Gharahdaghi et al. [15].

Each sample was suspended in 0.8 μl 0.5 g/l of matrix solution (a-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:1, *v*/*v*) acidified with 0.1% (*v*/*v*) trifluoroacetic acid). Then, the mixture was immediately spotted onto the stainless steel MALDI target plate, and allowed to dry and crystallize under room temperature. Mass spectrometry was performed using a 4700 Proteomics Analyzer (TOF/TOF^{TM}, Applied Biosystems, USA) equipped with a 337-nm Nd: YAG laser.

The proteins were identified by peptide mass fingerprinting and tandem mass spectrometry using the program MASCOT v 1.9 (Matrix Science, London, UK) against SWISS-PROT database with GPS explorer software (Applied Biosystems, USA).

Validation of Differentially Expressed Spots by Western Blotting

Western blotting was performed three times to confirm differential expression of the proteins. As it was indicated in the study performed by Schizas et al. [9] that type III collagen may be involved in regulating energy uptake during AT healing, this protein was finally selected for Western blotting. The protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed by anti-collagen III antibody

(Santa Cruz Biotechnology, CA, USA), and the proteins were normalized with anti-β-actin antibody (Santa Cruz Biotechnology, CA, USA) and visualized employing the Pierce ECL detection system. The digital image was obtained by scanning the film and gray value analysis [16].

Results

Comparison of Two-Dimensional Electrophoresis of Proteins

The two-dimensional electrophoresis maps of the proteins are shown in Fig. 1. It was demonstrated that most of the protein spots were concentrated in the pH 3.5–9 region. A total of 463±12, 511±39, and 513±80 protein spots were detected in the gels for the AT

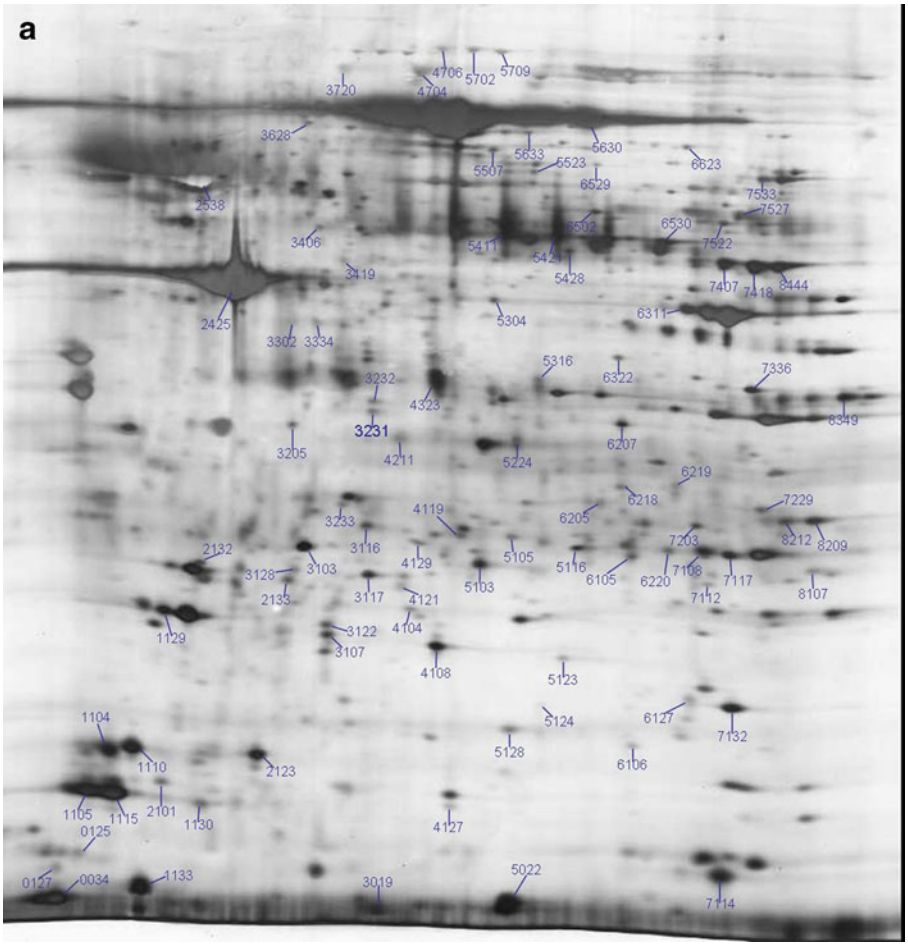

Fig. 1 Two-dimensional electrophoresis maps of the proteins for AT samples of rabbits in different groups 21 days following the AT microsurgery: **a** group A (control group), **b** group B (immobilization group), **c** group C (early motion group)

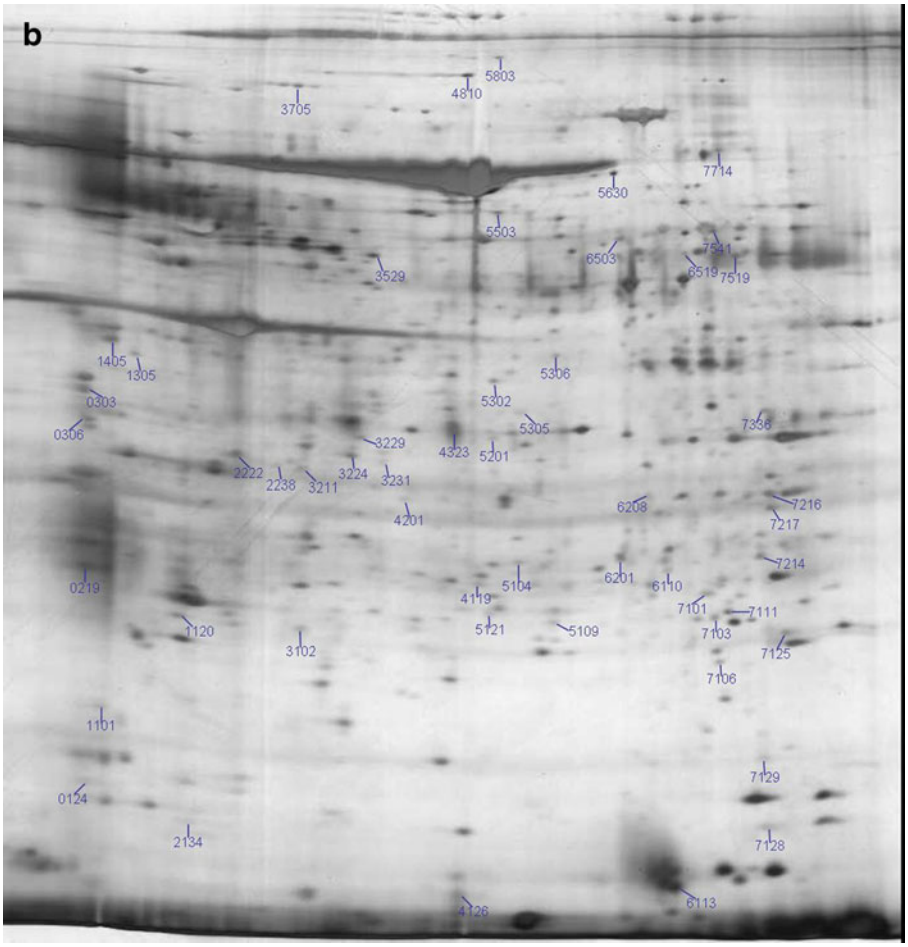

Fig. 1 (continued)

samples of the rabbits in group A (control group), group B (immobilization group), and group C (early motion group), with a total of 15, 8, 9 unique protein spots obtained in each group, respectively, as shown in Table 1.

Identification of Differentially Expressed Proteins and Bioinformatics

The proteins with expression abundance difference of up to five times for the AT samples between two groups were considered to be differentially expressed proteins. According to this criterion, many differentially expressed proteins were identified in group A (control group), group B (immobilization group), and group C (early motion group), as shown in Tables 2, 3, and 4. Some of the proteins are involved in various metabolism pathways and may play an important role in AT healing, e.g., glycerol-3-phosphate dehydrogenase, annexin A2, heat shock 27 kDa protein 1 (HSPB1), alpha-crystallin B chain-golden hamster, etc. Figure 2 shows the identification result of peptide 2222 detected in the early motion group, and this peptide was identified as alpha-1 type III collagen. Those

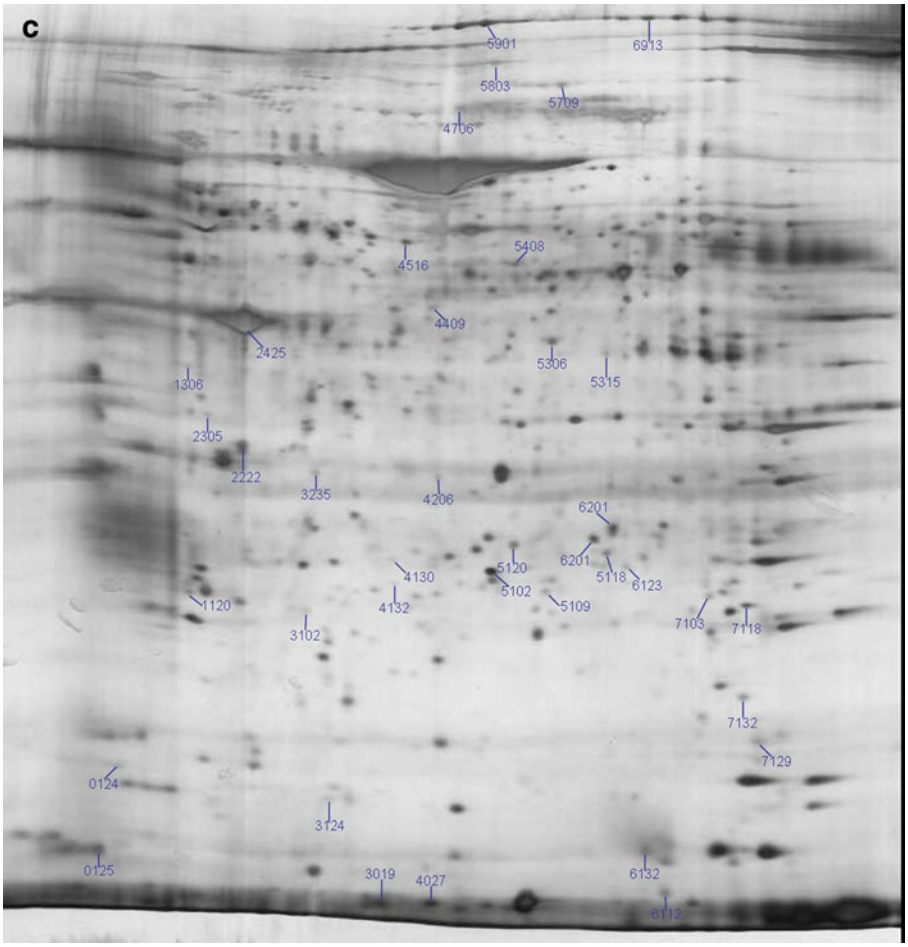

Fig. 1 (continued)

unidentified spots were considered as protein mixtures and were further separated and identified.

Western Blotting

The selected protein, alpha-1 type III collagen, was validated through Western blotting. A total of seven samples were used in each of the three groups, and repeated tests were performed three times. Figure 3 shows the results of Western blotting, displaying the comparison of alpha-1 type III collagen (species, rabbit) and β-actin for group A (control group), group B (immobilization group), and group C (early motion group), and the relative protein expression of alpha-1 type III collagen. It was indicated that the expression abundance of alpha-1 type III collagen in the early motion group was significantly higher than that of the control group and the immobilization group, and this difference was significant through a one-way analysis of variance ($P<0.01$). These data agreed with the results obtained through the two-dimensional electrophoresis.

Table 1 Detection of protein spots in the two-dimensional polyacrylamide gels for the Achilles tendon samples of the rabbits in group A (control group), group B (immobilization group), and group C (early motion group)

Groups	Protein spots in the two-dimensional gel	Matched protein spots	Matching rate (%)
Group A (1)	475	470	98.8
Group A (2)	461	458	99.3
Group A (3)	452	449	99.3
Group A (mean value)	463±12	459±11	99.1
Group B (1)	556	556	100
Group B (2)	490	488	99.6
Group B (3)	487	485	99.6
Group B (mean value)	511±39	510±40	99.7
Group C (1)	506	498	98.4
Group C (2)	512	508	99.2
Group C (3)	521	516	99.0
Group C (mean value)	513±80	508±90	98.8

Discussion

Postoperative early motion is nowadays generally accepted as a method of choice to treat AT rupture in addition to the introduction of new surgical techniques [17, 18]. However, a mechanobiological insight into the contribution of postoperative early motion to healing of AT rupture has not been achieved as yet. Proteomic analysis can be used as a potential useful tool to elucidate the rationale behind the success of postoperative early motion treatment, but a literature survey has shown that research in the area of proteomic analysis of AT is lacking [19]. In the present study, a study of proteomic analysis of differential protein expression in ruptured AT of rabbits treated with two different methods, i.e., postoperative cast immobilization and postoperative early motion, was performed using two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Additionally, one of the differentially expressed proteins, i.e., alpha-1 type III collagen, was validated through Western blotting. It was demonstrated from the analysis of the identified proteins in group A (control group), group B (immobilization group), and group C (early motion group) that some of the proteins may contribute to healing of AT rupture, especially for those detected in the early motion group such as HSPB1 and annexin A2.

HSPB1 is a stress response protein and shows dramatically increased expression in response to cellular stress. The postoperative early kinesitherapy or centrifugal stress may stimulate the expression abundance as identified in group C (early motion group). It also plays a role in signal transduction. HSPB1 protects against ischemic and reoxygenation-mediated injury and inhibits apoptosis induction [20]. Our experimental findings support the literature results. In addition, HSPB1 functions as an adenosine triphosphate-independent molecular chaperone and is involved in the remodeling of cytoskeleton during embryogenesis and protection of the cytoskeleton in cells exposed to various stresses. HSPB1 phosphorylated by p38 MAP kinase is necessary for migration of vascular smooth muscle cells, neutrophils, fibroblasts, and breast epithelial cells [21]. HSPB1 is also thought to be a collagen-binding chaperone involved in the maturation of collagen, its phosphorylation correlated with both fibroblast-populated collagen lattice model contraction and wound contraction in rats in vivo. These findings appear to be clinically relevant since wound contraction represents a significant factor in closing an open wound. On the contrary, the numerous defects in the function of HSPs associated with diabetes could

Table 2 Identification of differentially expressed proteins for the AT samples of the rabbits in group A (control group)

No.	Spot no.	NCBI index code	Protein Name	Protein score	Best ion C.I. (%)	MW (Da)	pI	Functional association
1	5,128[a]	gi|16550741	Unnamed protein product	72	61	50,280.2	5.44	Chromosome segregation ATPases
2	7,624[a]	gi|73957867	Procollagen C-proteinase enhancer protein precursor	150	100	48,555.2	6.82	Type I procollagen COOH-terminal proteinase enhancer
3	6,322[a]	gi|67967759	F-actin capping protein alpha subunit	164	100	33,015.4	5.56	*Homo sapiens* capping protein (actin filament) muscle Z-line
4	5,304[b]	gi|44889024	Serum albumin precursor	152	100	68865.3	5.85	Binds various cations, fatty acids and bilirubin
5	5,103[b]	gi|109013292	Glutathione *S*-transferase M4 isoform 9	138	100	25,750.2	7.68	Cytosolic dimeric proteins
6	6,207[b]	gi|3043365	Glycerol-3-phosphate dehydrogenase, cytoplasmic	90	99.955	37,585.4	6.32	Energy production and conversion
7	7,108[c]	gi|73997312	Triosephosphate isomerase isoform 3	69	71.453	19,575.1	7.66	Glycolytic enzyme
8	3,205[c]	gi|75069379	Annexin A8	137	100	36,656.5	5.53	An anticoagulant protein
9	8,209[c]	gi|115461	Carbonic anhydrase 3	90	99.962	29,361.8	7.84	Reversible hydration of carbon dioxide
10	3,107[c]	gi|73986005	Tetranectin precursor	77.2	68.876	26,515.4	8.53	C-type lectin/C-type lectin-like domain
11	3,116[c]	gi|6671549	Peroxiredoxin 6	280	100	24,811.0	5.98	Homodimeric thiol-specific antioxidant proteins

[a] The unique proteins identified for the AT samples in group A (control group

[b] The proteins with the expression abundance in group A (control group) five times higher than that of group B (immobilization group)

[c] The proteins with the expression abundance in group A (control group) higher than that of Group B (immobilization group) and five times higher than that of group C (early motion group)

Table 3 Identification of differentially expressed proteins for the AT samples of the rabbits in group B (immobilization group) at 21 days postoperation

No.	Spot no.	NCBI index code	Protein name	Protein score	Best ion C.I. %	MW (Da)	pI	Functional association
1	5,503[a]	gi|44889024	Serum albumin precursor	83	71	68,865.3	5.85	Binds various cations, fatty acids, and bilirubin
2	4,323[a]	gi|75073382	Mimecan precursor	197	100	45,893.9	5.93	Induces bone formation in conjunction with TGF-beta-1 or TGF-beta-2 (by similarity)
3	5,325[a]	gi|1703316	Annexin A1	180	100	38,710.9	6.28	Signal transduction, calcium ion binding
4	6,201[b]	gi|999627	Chain B, Porcine E-Trypsin (E.C.3.4.21.4)	87	76	8,813.5	6.67	Trypsin-like serine protease
5	3,705[b]	gi|74007216	Melanoma antigen family B, 4	114	98	8,865.7	10.08	Transcriptional control
6	1,405[b]	gi|1311496	Transcriptional regulator	85	76	8,865.7	9.92	Transcriptional regulation
7	2,682[c]	gi|74204751	Unnamed protein product	94	99.984	39,957.2	6.4	Fibrillar collagen C-terminal domain
8	3,529[c]	gi|601905	Alpha-1-antitrypsin	123	100	38,802.7	5.94	Serine protease inhibitors
9	7,214[c]	gi|164840	Carbonic anhydrase I	120	100	25,677.1	8.0	Catalyze the reversible hydration of carbon dioxide in a two-step mechanism
10	7,111[c]	gi|73981675	Proteasome subunit alpha type2 (proteasome component C3)	173	100	20,127.6	9.78	The central enzyme of nonlysosomal protein
11	6,417[c]	gi|77735571	Asporin precursor	116	99.999	42,092.2	9.24	Participate in protein–protein interactions and cellular locations
12	4,405[c]	gi|109149722	Similar to 60S acidic ribosomal protein P0 (L10E)	101	99.962	30,223.9	8.68	Associated with GTPase activities in protein synthesis

[a] The proteins with the expression abundance in group B (immobilization group) five times higher than that of group C (early motion group)

[b] The proteins with the expression abundance in group B (immobilization group) five times higher than that of group A (control group)

[c] The proteins with the expression abundance in both group B (immobilization group) and group C (early motion group) five times higher than that of group A (control group)

Table 4 Identification of differentially expressed proteins for the AT samples of rabbits in group C (early motion group) in 21 days postoperation

No.	Spot no.	NCBI index code	Protein name	Protein score	Best ion C.I. %	MW (Da)	pI	Functional association
1	4,516[a]	gi|47523848	Dihydrolipoamide *S*-succinyltransferase	140	100	48,945.6	9.0	Play a key role in redox regulation
2	2,312[a]	gi|118627572	F-actin capping protein subunit alpha 1	176	100	32,996.4	5.53	Regulate the polymerization state of actin
3	6,203[a]	gi|115523801	Transcriptional regulator, LysR family	90	99.48	32,254.5	10.21	Transcriptional regulator
4	4,807[a]	gi|5732934	Pro-alpha 1 type 1 collagen	105	99.985	20,413.1	9.26	Tissue regulation
5	7,339[b]	gi|63594732	Annexin A2 (Annexin II)	174	100	32,578.6	5.95	Signal transduction and calcium ion binding
6	5,102[b]	gi|50979116	Heat shock 27 kDa protein 1	124	100	22,924.7	6.23	Stress resistance and actin organization
7	5,120[b]	gi|74149249	Unnamed protein product	74	52	71,728	9.27	General transcription factor III C 1
8	2,222[b]	gi|180414	Alpha-1 type III collagen	117	100	36,407.8	6.21	Fibrillar collagen C-terminal domain
9	5,709[b]	gi|74008821	Filamin 1 (endothelial actin-binding protein) (actin-binding protein 280)	179	100	278,285.7	5.78	Regulation of cell shape (cortexillins), and signaling proteins (Vav)
10	7,132[b]	gi|2119188	Alpha-crystallin B chain-golden hamster (fragment)	136	100	19,709	6.76	Effects on the apoptotic pathway and on metastasis
11	5,320[b]	gi|67940425	Peptidoglycan glycosyltransferase	67	89	77,976.5	8.92	Formation of the glycan chains of the cell wall
12	7,412[b]	gi|119512191	1-Acyl-sn-glycerol-3-phosphate acyltransferase	154	138	23,276.4	9.66	Carbohydrate transport and metabolism
13	5,213[b]	gi|3789966	Fibrinogen A-alpha chain	147	139	41,211.7	6.16	Platelet aggregation and coagulation of blood
14	5,306[c]	gi|73980918	Macrophage capping protein	92	87	38,802.7	6.04	Actin capping
15	5,115[c]	gi|94369185	Unnamed protein product	96	85	28,813.9	6.67	Glycolysis, similar to phosphoglycerate mutase 1
16	7,118[c]	gi|27710302	Zinc finger and SCAN domain-containing protein 12 (zinc finger protein 29 homolog)	147	124	20,094.4	9.37	May be involved in transcriptional regulation

[a] The unique proteins identified for the AT samples in group C (early motion group)

[b] The proteins with the expression abundance in group C (early motion group) five times higher than that of group B (immobilization group)

[c] The proteins with the expression abundance in group C (early motion group) five times higher than that of group A (control group)

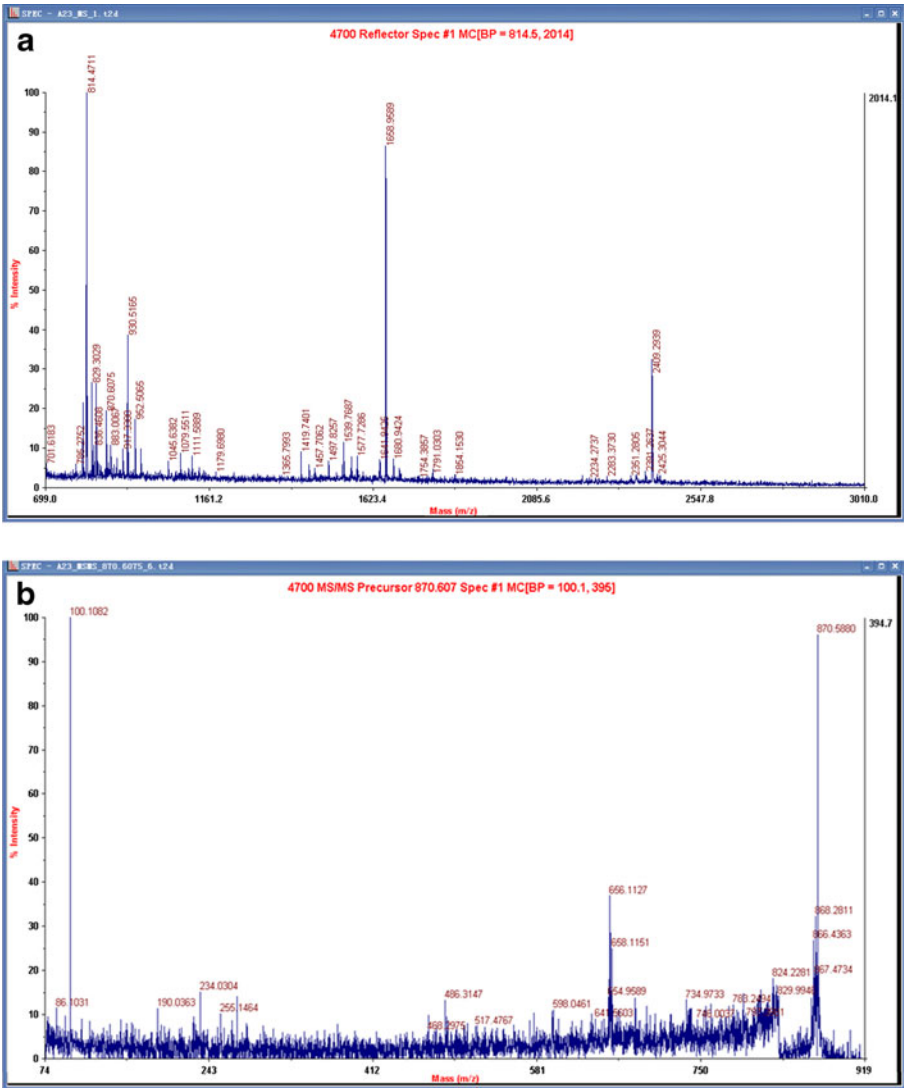

Fig. 2 Identification result of peptide 2222 detected for AT samples of rabbits in the early motion group: **a** peptide mass fingerprinting, **b** mass spectrometry sequence analysis. The peptide was identified as alpha-1 type III collagen

contribute to the commonly observed complications and delayed wound healing in diabetics. However, its plausible role in wound healing remains unknown [22].

Annexin A2, as a calcium-dependent phospholipid-binding protein, plays a role in regulation of cellular growth and in signal transduction pathways. It is also involved in a number of biochemical processes, including cell proliferation, ion channel activation, and cell–cell interactions. The presence of Annexin A2 in the samples of the early motion group could facilitate proliferation of tendon cells and an increase in the circumference of collagen fibers. This also means that Annexin A2 could be involved in many normal and pathological processes which accelerate AT healing. The abundance of the two proteins in the early motion group was

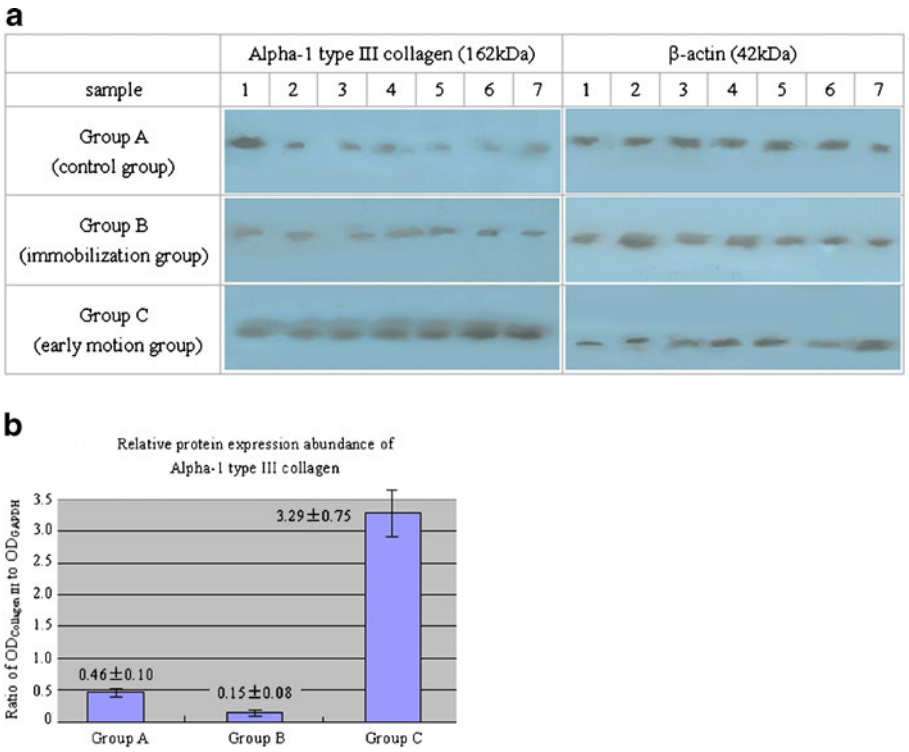

Fig. 3 Western blotting results of proteins for the three different groups: **a** distribution of alpha-1 type III collagen and β-actin for the seven samples in each group, **b** comparison of expression abundance of alpha-1 type III collagen

significantly higher than that in the immobilization group. As the AT microsurgery for the rabbits in these two groups was the same and the only difference was the postoperative treatment method following microsurgery, the significant increase in abundance of HSPB1 and Annexin A2 is likely due to the application of postoperative early motion treatment.

The two proteins may stimulate the same signaling pathway that induced alpha-1 type III collagen expression abundance. The presence of alpha-1 type III collagen could contribute to energy adjustment involved in the AT making the tendon more viscoelastic, and it was considered that the immobilization treatment to some extent restrained the generation of alpha-1 type III collagen [9]. This protein was found to be abundantly expressed in group C (early motion group). It indicates that alpha-1 type III collagen plays an important role for the AT healing.

Previous research has shown that postoperative early centrifugal stress could promote an overall reconstruction of the ruptured AT through a mechanobiological mechanism, and the time when tendon mobilization is initiated after surgery is an important factor, e.g., immediate mobilization was superior to delayed mobilization in terms of ultimate strength in healing canine flexor tendons [23, 24]. The immediate mobilization is greater in terms of the diameters of collagen fibrils ($P<0.01$) to cast immobilization in healing postoperative 21 days of rabbits AT, and fibroblasts are more maturate, collagen fibers are distributed more regularly in the mobilization group [25], indicating the restoring of the functional properties of the tendons more rapidly than cast immobilization of an identical surgical repair. While an insight into the rationale was not obtained at the time, we demonstrated in the present study that the

differentially expressed proteins, e.g., the mechanical stimulation protein HSPB1, may play a key role in this process. On the contrary, postoperative cast immobilization treatment resulted in a situation where the tendon was in a tension-free or disused state or just like in diabetic state, and healing of AT rupture was to a certain degree restrained due to this reason.

Although the use of proteomic analysis in revealing the nature of changes in a biological way has been available in many studies [11, 16], research in the area of treatment of AT rupture based on the analysis of differentially expressed proteins has rarely been reported. One typical research in this area was the study performed by Harris et al. in 2003, in which localized tendon healing was investigated through quantifying protein expression [19]. By employing an isotope-coded affinity tag analysis, they could detect subtle differences in protein levels which provided a detailed picture of tendonitis healing. However, Achilles tendonitis was induced by injection of collagenase in their study; thus, the research actually did not investigate the influence of different methods in treating AT rupture on a protein level, which was the main aim of the present study. While it was considered an original study of proteomic analysis in the treatment of AT rupture, some differentially expressed proteins were not successfully identified in this study, which may further help gain a deep insight into the contribution of postoperative early centrifugal stress to healing of AT rupture at 21 days postoperation. Therefore, further research is required to investigate this issue by using other methods to identify these proteins and their signaling pathway during the process of AT healing.

Conclusions

Differentially expressed proteins were detected in AT samples of rabbits treated with postoperative early kinesitherapy, which were considered to promote healing of Achilles tendon rupture. This research provides an initial study in proteomic analysis of AT rupture at 21 days postoperation to gain insight into the mechanobiological mechanism of postoperative early centrifugal stress to the overall reconstruction of ruptured AT at 21 days postoperation. According to our study here, early postoperative motion can promote healing of rabbit Achilles tendon tissue.

Acknowledgments This study was financially supported by the National Natural Science Foundation of China (Grant No. 30760256) and The First Teaching Hospital of Xinjiang Medical University (Grant No. 2002-XYQD-03, 2005- XYQD-03). Many thanks are due to Hong Jin (PhD, Associate Professor), Pengyuan Yang (PhD, professor), Fenying Yang (senior lab technician), and Xinwen Zhou (senior lab engineer), Centre of Proteomics and Systems Biology, Institutes of Biomedical Sciences, Fudan University, Shanghai, for 2D polyacrylamide gel electrophoresis. We also thank Yuanting Zhang, Binyin Zhang, Xiulian Yin (senior lab assistant), Institutes of Biomedical Sciences, Tsinghua University, Beijing, for Western blotting.

Competing Interest None to declare.

References

1. Carden, D. G., Noble, J., Chalmers, J., et al. (1987). Rupture of the calcaneal tendon: the early and late management. *J Bone Joint Surg Br, 69*, 416–420.
2. Lee, D. K. (2007). Achilles tendon repair with acellular tissue graft augmentation in neglected ruptures. *The Journal of Foot and Ankle Surgery, 46*, 451–455.
3. Maffulli, N., & Scotland, A. (1999). Current concepts review: rupture of the Achilles tendon. *J Bone Joint Surg Am, 81*, 1019–1036.

4. Cetti, R., Christensen, S. E., Ejsted, R., et al. (1993). Operative versus nonoperative treatment of Achilles tendon rupture: a prospective randomized study and review of the literature. *The American Journal of Sports Medicine, 21*, 797–799.
5. Möller, M., Movin, T., Granhed, H., et al. (2001). Acute rupture of tendo Achilles: a prospective randomized study of comparison between surgical and non-surgical treatment. *Journal of Bone and Joint Surgery (British Volume), 83*, 843–848.
6. Speck, M., & Klaue, K. (1998). Early full weight bearing and functional treatment after surgical repair of acute Achilles tendon rupture. *The American Journal of Sports Medicine, 26*, 789–793.
7. Uchiyama, E., Nomura, A., Takeda, Y., Hiranuma K., & Iwaso H. (2007). A modified operation for Achilles tendon ruptures. *The American Journal of Sports Medicine, 35*(10), 1739–1743.
8. Jiasharete, J. (2003). Review article: development of suture method to repair Achilles tendon rupture. *Chinese Journal of General Practice, 2*, 49–53.
9. Schizas, N., Li, J., Andersson, T., et al. (2010). Compression therapy promotes proliferative repair during rat Achilles tendon immobilization. *Journal of Orthopaedic Research, 28*, 852–858.
10. Jiasharete, J., & Bai, J. P. (2008). Mechanobiology during Achilles tendon healing. *Chinese Journal of Clinical Rehabilitative Tissue Engineering Research, 12*, 8352–8357.
11. Wu, Y. F., Yin, Z. Z., & Jiasharete, J. (2009). Proteomics and its application in the studies of orthopedic disease. *Chin J Med Postgrad, 7*, 758–761.
12. Yang, S. Q. (1998). *Hygiene statistics*. Beijing: People's Medical Publishing House.
13. Jiasharete, J., Bai, J. P., Darebai, R., et al. (2010). Factors influencing the tensile strength of repaired Achilles tendon: a biomechanical experiment study. *Clinical biomechanics, 25*, 789–795.
14. Yan, J. X., Wait, R., Berkelman, T., et al. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis, 21*, 3666–3672.
15. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., et al. (1999). Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis, 20*, 601–605.
16. Sun, Q. J., Miao, M. Y., Jia, X., et al. (2008). Subproteomic analysis of the mitochondrial proteins in rats 24h after partial hepatectomy. *Journal of Cellular Biochemistry, 105*, 176–184.
17. Mandelbaum, B. R., Myerson, M. S., & Forster, R. (1995). Achilles tendon ruptures: a new method of repair, early range of motion, and functional rehabilitation. *The American Journal of Sports Medicine, 23* (4), 392–395.
18. Motta, P., Errichiello, C., & Pontini, I. (1997). Achilles tendon rupture: a new technique for easy surgical repair and immediate movement of the ankle and foot. *The American Journal of Sports Medicine, 25*, 172–176.
19. Harris, R. D., Nindl, G., Balcavaqe, W. X., et al. (2003). Use of proteomics methodology to evaluate inflammatory protein expression in tendinitis. *Biomedical Sciences Instrumentation, 39*, 493–499.
20. Cohly, H. H. P., Scott, H., Ndebele, K., et al. (2002). Differential gene expression of fibroblasts: keloid versus normal. *International Journal of Molecular Sciences, 3*, 1162–1176.
21. Salinthone, S., Tyagi, M., & Gerthoffer, W. T. (2008). Small heat shock proteins in smooth muscle. *Pharmacology and Therapeutics, 119*, 44–54.
22. Atalay, M., Oksala, N., Lappalainen, J., et al. (2009). Heat shock proteins in diabetes and wound healing. *Current Protein & Peptide Science, 10*, 85–95.
23. Wang, J. (2006). Mechanobiology of tendon. *Journal of Biomechanics, 39*, 1563–1582.
24. Gelberman, R. H., Manske, P. R., Akeson, W. H., et al. (1986). Flexor tendon repair. *Journal of Orthopaedic Research, 4*, 119–128.
25. Bin, T., Bai, J. P., Jiasharete, J., et al. (2010). The histological effects of early mobilization in the healing after achilles tendon of rabbits rupture repair. *Journal of Chinese Physician, 12*, 19–22.