

Loadless Tendon Explant Model Exhibits Spontaneous Degradative Phenotypes: An *In Vitro* Study of Human Tendons

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Abstract

Background: Although the tendon explant model has been gradually recognized for simulating tendon characteristics *in vitro*, there is still no consensus on a standardized culture protocol. Recently, tendon explants have been applied to senescence-related research but whether their own changes affected the credibility of the conclusions was not confirmed. Therefore, this study aimed to systematically evaluate the reliability of the loadless tendon explant model by characterizing its dynamic macroscopic morphological and molecular biological changes during culture.

Methods: This study collected surgical samples from patients as loadless tendon explants for culture and conducted molecular biology and cytology tests once every 3 days within 15 days. Both their macroscopic and microscopic changes were recorded and analyzed, with each compared to the corresponding parameters at day 0.

Results: Loadless tendon explants spontaneously underwent progressive disintegration, leading to the collapse of the original dense structure. Consistently, the collagen fibers also gradually became loose and fractured, contributing to a significant decrease of collage density from day 9. In addition, the senescence and apoptosis markers P21 and Caspase 3 increased over time, and there were statistically significant differences starting from day 6, respectively. The main intrinsic component, Collagen I, and the main catabolic enzyme, metalloproteinase 13 (MMP 13), could hardly be detected since day 3.

During the daily evaluations from day 0 to day 4, Collagen I gradually decreased and was barely detectable by day 3, whereas MMP 13 increased on day 1 followed by a rapid decline since day 2.

Conclusions: These results indicate that loadless tendon explants rapidly degrade and lose matrix integrity, with their spontaneous changes compromising the reliability of research conclusions related to senescence and apoptosis. Therefore, further optimization is still required before a well-established protocol is applied.

Keywords: Tendons; Collagen; Tissue transplantation; Senescence; Apoptosis

Introduction

Tendinopathy is a chronic tendon disease that has not been precisely defined, characterized by pain, limited function, and reduced exercise capacity. It is susceptible among athletes, manual laborers, the elderly, and obese individuals [1]. In the past three decades, the global incidence of tendinopathy has increased by 58% and it already accounts for 30-50% of all outpatients with musculoskeletal issues [2]. However, due to the unclear pathogenesis and complex pathological features of tendinopathy, there is no consensus on its treatment, which mainly includes pain management and physical therapy lasting for months or even years [3]. This imposes a huge mental and economic burden on patients and may potentially lead to heterogeneity in therapeutic efficacy. Therefore, exploring the mechanisms of tendinopathy is a prerequisite for an effective solution, and a reasonable experimental method is of first priority. Nevertheless, the dense structure of tendon tissue, its susceptibility to mechanical loading, and its requirement for a relatively hypoxic environment make it difficult to simulate the authentic state of tendon tissue *in vitro* [4-6]. Additionally, the spontaneous alterations of tendon tissue during culture may interfere with the results [7]. Thus, clarifying the biochemical and histological changes of tendon tissue during *in vitro* culture should precede the experiment, which is beneficial for ensuring the accuracy of conclusions.

The theoretical basis for *in vitro* tendon culture can be traced back to Bi et al, who successfully isolated and extracted

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tendon stem/progenitor cells from human and murine tendon tissues and confirmed their cellular stemness [8]. Currently, the tendon explant model based on intact tissue has been acknowledged by satisfied simulation of the *in vivo* situation of tendons, which helps to evaluate cell status and tissue characteristics [9, 10]. For example, the latest attractive research has applied it to the *in vitro* study of tendon aging [11]. However, the lack of a comprehensive evaluation of this model leads to uncertainties in the culture method and may affect the exactness of research. Previous studies merely focused on a few aspects such as collagen-related or proliferation gene expressions [12]. Therefore, a time-series analysis of the cellular and matrix changes of tendon explant model is necessary for relevant studies. Especially, research on human tendon explants will accelerate the possibility of clinical translation.

Thus, we intended to harvest the redundant parts of tendons discarded after autologous tendon reconstruction surgery from several patients for tendon explant culture. At different time points (day 0, day 3, day 6, day 9, day 12, day 15), cellular and molecular analyses of these samples were carried out by tissue staining and molecular biology techniques. Then the changes of collagen fibers, P21, Caspase 3, Collagen I, and matrix metalloproteinase 13 (MMP 13) over time were quantitatively evaluated. Collectively, this study assessed the feasibility of the existing tendon explant culture method, provided a theoretical basis for the improvement of those methods, and promoted the development of mechanism research on tendinopathy. The conclusion would benefit the basic research and clinical application in the use of human tendon explants for the tendinopathy treatment.

Materials and Methods

Ethical declaration

Our research was conducted in compliance with the ethical standards of the responsible institution on human subjects as well as with the Helsinki Declaration. This study was approved by the Medical Ethics Committee of the Clinical Research Ethics Committee of Shenzhen Second People's Hospital (2025-041-01PJ). All participants provided written consent for this study prior to the experiment.

Tendon explant management and culture

We enrolled volunteer patients who underwent autologous tendon reconstruction due to anterior cruciate ligament rupture. The specific inclusion and exclusion criteria were described.

Inclusion criteria

1) Tendon samples are obtained from the semitendinosus muscle. 2) Aged 18 - 60 to exclude physiological degeneration-related tendon quality differences. 3) No obvious macroscopic damage or degeneration of the semitendinosus tendon (e.g.,

no tear, calcification, fibrosis, abnormal color). 4) Preoperative magnetic resonance imaging (MRI) confirms normal structure of the semitendinosus tendon without organic lesions.

Exclusion criteria

1) History of systemic diseases: obesity (body mass index (BMI) ≥ 30 kg/m²), type 2 diabetes, hyperlipidemia, hypothyroidism, rheumatoid arthritis, hyperthyroidism, hyperparathyroidism, chronic renal failure, systemic lupus erythematosus, severe liver insufficiency, ankylosing spondylitis, and malignant tumors. 2) History of tendon-related diseases (e.g., tendinitis, tendon tear, calcification, and enthesopathy). 3) Trauma or surgical history at the sample site within the past 6 months. 4) History of infection at the sample site or systemic infection. 5) Long-term (past 3 months) use of glucocorticoids, immunosuppressants, chemotherapeutic drugs, etc. 6) Long-term smoking (≥ 10 cigarettes/day for ≥ 5 years) or excessive alcohol consumption (≥ 25 g alcohol/day).

Five male patients aged 24 - 46 participated. The discarded tendons during the surgery were collected for culture, histological staining, and molecular biology experiments. They were immersed in low-glucose Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin [11]. Each of them was evenly and sterilely divided into 12 parts. Then they were randomly distributed on a plate, with 2 mL of culture medium added. Subsequently, they were cultured at 37 °C, 5%CO₂, and without mechanical load. Medium was replaced every 24 h. We also harvested tendons from another three male patients aged 30 - 36 to perform subsequent protein quantitation every day. The collection and culture methods were the same as the previous ones.

Morphological observation

We performed sampling and recording on day 0/3/6/9/12/15. At each time point, two samples were collected from each patient. Each sample was washed three times with double-distilled water to remove surface impurities. Photographs were taken at different time points.

Histological staining

To investigate the collagen content, cellular senescence, and apoptosis of tendon explants, five independent samples at each time point were immersed in 4% paraformaldehyde (PFA) (Servicebio, G1101, Wuhan, China) at room temperature for more than 48 h. Then, 5 μ m paraffin slices were prepared.

We used the kit (Solarbio, G1340, Beijing, China) to perform the Masson staining. The slices were stained with Ponceau-Fuchsin for 5 min, then washed with 1% phosphomolybdic acid for 1 min. Subsequently, they were stained with Aniline Blue for 2 min and washed with 1% ethanoic acid for 1 min.

For the immunohistochemical staining, the slices were

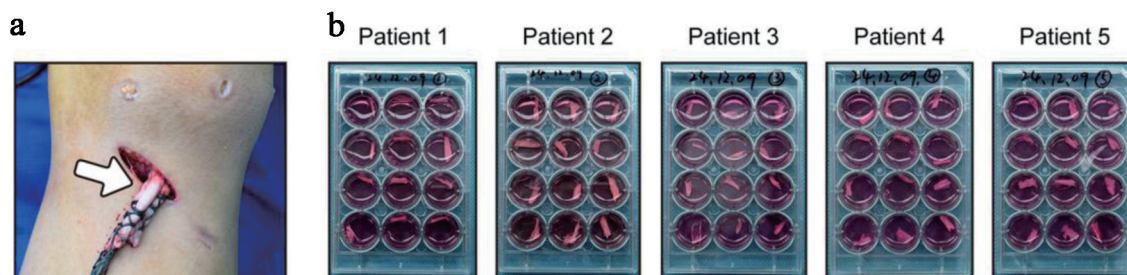


Figure 1. Redundant tendon tissues were intraoperatively harvested and cultured without load *in vitro*. (a) Redundant tendon tissues were intraoperatively harvested from patients after anterior cruciate ligament reconstruction. (b) Tendon tissues were divided into 12 equal parts and cultured without load.

heated in sodium citrate buffer (pH = 6.0) at 95 °C for 20 min to complete antigen retrieval. After cooling to room temperature, they were incubated with 3% H₂O₂ for 10 min, followed by incubation with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 30 min. Subsequently, they were incubated overnight at 4 °C with diluted P21 polyclonal antibody (Proteintech, 10355-1-AP, 1:200, Wuhan, China) or Caspase 3 polyclonal antibody (Proteintech, 19677-1-AP, 1:200, Wuhan, China). After washing with PBS, they were incubated with Goat anti-rabbit IgG H&L (HRP) secondary antibody (Abcam, ab6721, 1:1,000, Cambridge, UK) at room temperature for 1h, and then developed with 3,3-diaminobenzidine (DAB) (Beyotime, P0203, Shanghai, China).

All slices were routinely dehydrated, cleared, and mounted for imaging. The percentage of collagen fiber area and positive cells were analyzed using ImageJ software. 1) Collagen fiber density. RGB images of sections were opened, and the blue-stained collagen fibers were selectively segmented via the “Threshold” function by adjusting blue channel parameters. After confirming the selected collagen regions, the “Set Measurements” function was activated with the “Area” and “Limit to threshold” options enabled. Collagen fiber area was measured by pressing the “M” key, and the total image area was calculated as the product of image width and height in pixels. The collagen fiber density was finally computed as (collagen fiber area/total image area) × 100%. 2) Positive cell rate. RGB images of sections were imported, and DAB-positive cells (brown signal) were segmented via the “Threshold” function by adjusting red/green channel parameters to exclude background. Positive cells were automatically counted with “Analyze Particles” (size and circularity optimized by target cell morphology) after enabling “Cell Count” and “Limit to threshold” in “Set Measurements”. Total nucleated cells were quantified by selecting hematoxylin and eosin (H&E)-stained nuclei (blue) via threshold adjustment, and the positive cell rate was calculated as (number of DAB-positive cells/total H&E-stained nucleated cells) × 100%.

Western blot

To investigate the protein expression of anabolism and catabolism in tendon explants, five independent samples at each time point were lysed using radioimmunoprecipitation assay (RIPA,

Beyotime, P0013B, Shanghai, China). The protein concentration of the supernatant was determined by the bicinchoninic acid (BCA) method. Subsequently, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking the membrane with 5% skim milk, it was incubated overnight at 4 °C with Rabbit anti-Collagen I polyclonal antibody (Bioss, bs-0578R, 1:1,000, Beijing, China), or Rabbit anti-MMP 13 polyclonal antibody (Bioss, bs-10581R, 1:1,000, Beijing, China), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Proteintech, 60004-1-Ig, 1:50,000, Wuhan, China). Subsequently, the membranes were incubated with Goat anti-rabbit IgG H&L (horseradish peroxidase (HRP)) (Abcam, ab6721, 1:10,000, Cambridge, UK) or Rabbit anti-mouse IgG H&L (HRP) (Abcam, ab6728, 1:10,000, Cambridge, UK) secondary antibody at 37 °C for 2 h, and then developed with DAB (Beyotime, P0203, Shanghai, China). The other three independent samples were managed as the same.

Statistical analysis

SPSS 13.0 software was adopted. All data were presented as mean ± standard deviation (SD). Comparison was conducted by using repeated measures one-way analysis of variance (ANOVA), compared to day 0. $P < 0.05$ was considered statistically significant.

Results

Loadless tendon explants exhibited spontaneous collapse and disintegration

Firstly, we collected the redundant tendon tissues from five male patients aged 24 - 46 who underwent anterior cruciate ligament reconstruction on the same day (Fig. 1a). Regarding that the potential influences of obesity, inflammation, nutrition, and metabolites, we analyzed their baseline data. The results showed that their BMI, white blood cell count, platelet count, hemoglobin level, albumin level, creatinine level, and uric acid level were basically within the normal range (Table 1). Each tendon tissue was immediately divided into 12 equal

Table 1. Baseline Data of the Five Patients

	P1 (male)	P2 (male)	P3 (male)	P4 (male)	P5 (male)	Mean ± SD
Age (years)	24	30	31	35	46	33.2 ± 8.17
BMI (kg/m²)	27.62	24.07	21.01	26.42	24.21	24.67 ± 2.54
WBC (× 10 ⁹ /L)	7.53	5.61	10.07	9.40	7.82	8.09 ± 1.74
PLT (× 10 ⁹ /L)	1.91	1.81	2.35	2.30	2.33	2.14 ± 0.26
Hb (g/L)	161	144	134	155	144	147.6 ± 10.55
Alb (g/L)	42.5	41.4	43.6	41.9	41.8	42.24 ± 0.86
Cr (μmol/L)	69.0	108.1	95.6	72.8	72.0	83.5 ± 17.38
UA (mmol/L)	466.8	400.0	388.1	377.0	332.7	392.92 ± 48.51

Alb: albumin; BMI: body mass index; Cr: creatinine; Hb: hemoglobin; PLT: platelet count; SD: standard deviation; UA: uric acid; WBC: white blood cell count.

parts, and the loadless tendon explants were cultured *in vitro* as previously reported (Fig. 1b). To dynamically evaluate the macroscopic morphological changes of the tendon explants, we obtained images every 3 days within 15 days of the culture, so as to compare the macroscopic changes. The results showed that the tendon explants from all five patients exhibited spontaneous disintegration: as the culture time prolonged, the volume of the tendons gradually decreased, the surface became loose,

and the tendon fiber bundles separated (Fig. 2). In order to identify those changes from microscopic perspective, we performed Masson staining of the samples at each time point. The results showed that the internal collagen network collapsed over time, accompanied by density decrease and rupture of the local collagen fibers. Specifically, the collagen arrangement in the tendons on day 0 presented a dense parallel bundle structure, which was significantly disintegrated

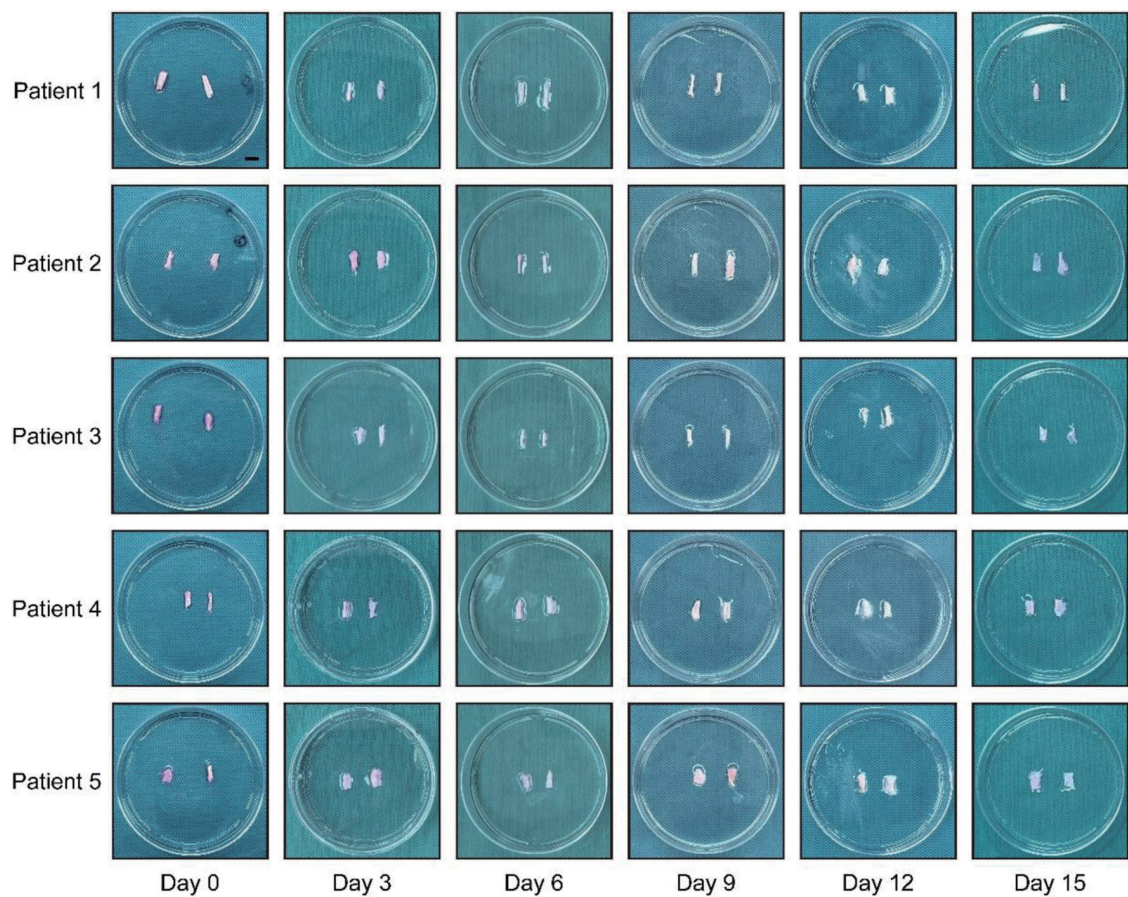


Figure 2. Loadless tendon explants underwent spontaneous disintegration. Images showing morphological changes of tendon explants on day 0, 3, 6, 9, 12, and 15 from the five patients, respectively. Scale bar = 1 cm.

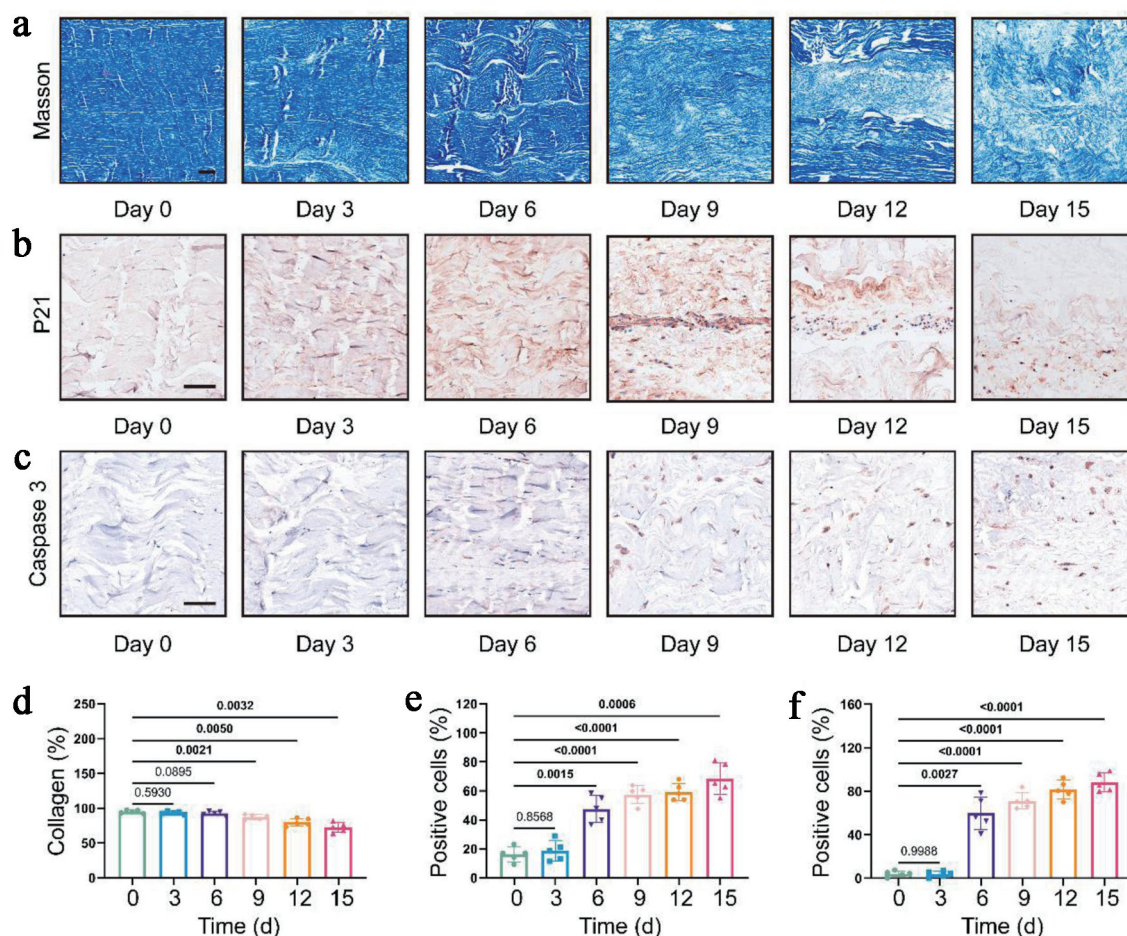


Figure 3. Cell and matrix in loadless tendon explants demonstrated degradative phenotypes. (a) Representative images of Masson staining at different time points. Scale bar = 100 μm. (b, c) Representative images of immunohistochemical staining of P21 and Caspase 3 at different time points, respectively. Scale bar = 100 μm. (d-f) Quantitative analysis of collagen fiber density (d), P21-positive cell rate (e), and Caspase 3-positive cell rate (f), respectively. Data were presented as mean ± SD (n = 5). Repeated measures one-way ANOVA (compared to day 0) were adopted for statistical analysis. The P values have been annotated accordingly, with values indicating statistical significance bolded. ANOVA: analysis of variance; SD: standard deviation.

by day 9 (Fig. 3a). The quantitative analysis showed that the collagen density of the tendon fibers gradually decreased. Compared to day 0, it decreased by approximately 10% on day 9, with statistical significance. Subsequently, the collagen density decreased by approximately 20% on day 15 (Fig. 3d).

Loadless tendon explants displayed cellular degradative phenotypes

Next, to evaluate the cell status of the tendon explants at various time points, we performed immunohistochemical staining for the senescence marker P21 and the apoptosis marker Caspase 3. The results showed that the number of positive cells for both markers increased significantly starting from day 6 (Fig. 3b, c). Quantitative analysis revealed that compared with day 0, the positive cell rate of P21 increased significantly from 15% to approximately 50%, and it was approximately 70% on day 15 (Fig. 3e). Similarly, the positive cell rate of Caspase 3

increased significantly from 5% to approximately 60%, and it was approximately 90% on day 15 (Fig. 3f).

Collagen I in loadless tendon explants decreased rapidly

Collagen I is the main structural component of the extracellular matrix of tendons, which significantly affects the stability of tendon tissues [13]. Its content usually accounts for more than 90% of the tissue [14]. To evaluate the compositional changes of tendon explants at various time points, we performed Western blot for it. The results showed that on day 0, Collagen I was highly expressed in the tendon tissues. However, its level decreased significantly on day 3 and could barely be detected at following time points (Fig. 4a). In addition, normal tendon tissues contain some MMP 13 to maintain tissue renewal, while the content increases under pathological conditions [15]. The results showed low-level MMP 13 expression on day 0 and day 3, and it could no longer be detected from day 6 to day 15 (Fig.

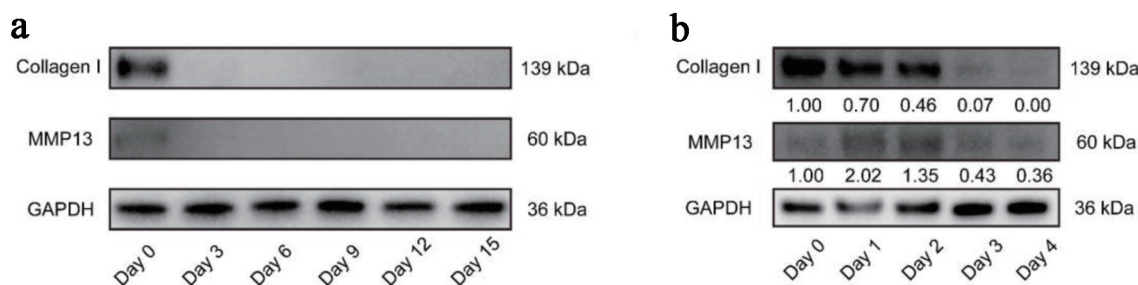


Figure 4. Western blot showing decreasing trend of Collagen I and MMP 13 expression in tendon explants over time. Representative images showing Collagen I and MMP 13 expressions from day 0 to day 15 (a) or from day 0 to day 4 (b). MMP: metalloproteinase 13.

4a). To elucidate their change pattern within the first few days, we harvested tendons from another three patients to carry out analysis daily. Consistently, Collagen I decreased gradually, especially from day 2 to day 3. Meanwhile, MMP 13 increased within 2 days but decreased dramatically since day 2 (Fig. 4b).

Discussion

To elaborate the pathogenesis of tendinopathy, previous studies have proposed that the method of tendon explants can help evaluate the cell state and tissue characteristics [16]. However, this method has not yet reached a consensus, and there is a lack of systematic and dynamic evaluation during the culture. Herein, we collected human tendons and analyzed them at cellular and molecular levels at different time points. The results revealed that loadless human tendon explants underwent structural disintegration and cellular degradative phenotypes, indicating that the method of tendon explants still requires reasonable optimization and improvement. Otherwise, their spontaneous alterations will ultimately affect the credibility of related studies.

Compared with previous studies, our study included the macroscopic morphological evaluation of loadless tendon explants and found the progressive tissue disintegration. This was consistent with the results reported by Wang et al. That is, after culture with load deprivation, tendons exhibit degenerative changes and weakened mechanical properties. Moreover, they found that regaining mechanical stimulation could rescue the pathological changes and mechanical properties of the tendons [17]. Thus, mechanical stimulation was crucial for maintaining the stability of tendon tissue.

The absence of the original mechanical microenvironment can simultaneously trigger cell cycle arrest and cell state alterations [18]. In our experiment, the cell senescence and apoptosis markers P21 and Caspase 3 continuously accumulated. This is in line with Stowe et al, whose results showed P21 expression increased during the period from day 0 to day 7 during tendon explant culture [11]. Similarly, Andarawis-Puri et al, who used Caspase 3 staining to quantify the positive cells in patellar tendon, found that the number on day 7 was higher than that on day 3 [19]. Interestingly, we observed transiently active proliferation in local areas during the period from day 6 to day 12. We speculated that tendon disintegration promoted the contact and substance exchange between cells and the cul-

ture medium. It may also be related to the activation of the compensatory metabolic pathways of cells [20, 21]. In addition, the increasing ratio of senescent or apoptotic cells slowed in the late stage. Through Masson staining, we suspected that this could attribute to the impairment of structural support, which impeded the cell to adhere to the matrix. This also suggested that the integrity of the tendon tissue structure was a prerequisite for cell survival. Regarding this conjecture, analyzing the cells in the supernatant may identify the responsible factors.

Collagen I is the main structural component of the tendon tissue and a decisive component of its function [22, 23]. In this experiment, Collagen I in loadless tendon explants was highly expressed on day 0 but was nearly undetectable from day 3. This is completely different from the situations verified in previous experiments, in which the expression of Collagen I increased [24]. At the same time, MMP 13 has a low-level expression to help maintain the normal renewal of tendon tissue [15]. Consistent with this, we detected its low-level expression on day 0, but it could hardly be detected from day 3. Despite the insufficiency of the existing evidence for attribution, all of them implied that significant catabolism occurred in loadless tendon explants from day 0 to day 3. These results indicated that the method in this study could not simulate the physiological state of tendon tissue *in vivo* due to the spontaneous changes throughout the culture.

We acknowledge the limitations of this study. The narrow sample may introduce biological bias and restrict generalizability to other demographics (e.g., older individuals, females, those with tendinopathy). Besides, our observational study has not yet proposed specific methodological optimization strategies, and targeted interventional investigations are warranted in subsequent research to address this gap.

Collectively, this experiment has elucidated that this loadless tendon explant demonstrates spontaneously catabolic state at both biochemical and cellular levels. We believe that this method requires optimization in multiple aspects. Firstly, the enlarged sample size and age stratification should be considered. Secondly, more intensive sampling points can be set up in the initial stage of culture, and highly sensitive detection techniques can be applied to clarify the changes of Collagen I and MMP 13. Thirdly, multiple markers related to stress, proliferation, etc., can be evaluated. Fourthly, mechanical load, oxygen concentration, and medium components can be re-

garded as control variables to mimic *in vivo* environment of tendons to the greatest extent. Through those improvements, tendon explants can be more promising in exploring the tendinopathy mechanism.

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None to declare.

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Conflict of Interest

All contributing authors declare no conflict of interest.

Informed Consent

All tendon samples utilized in this study were obtained from the Biobank of Shenzhen Second People's Hospital, where donors had previously provided broad written informed consent allowing the use of their samples and associated anonymized data for future medical research. The current study protocol falls within the scope of this original consent. Informed consent was obtained from the subjects involved in the study.

Author Contributions

JWG and BSC took the lead in writing the manuscript. JWJ and YJY designed experiments and analyzed data. BSC and YLW was responsible for collecting tendon specimens and experimental data. SYJ collected data. JWJ and WCL conceived the study, secured funding, and supervised overall direction. All authors contributed critical feedback that shaped the study and manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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