







RESEARCH ARTICLE

Effects of Chemical Sterilization and Gamma Irradiation on the Biochemical and Biomechanical Properties of Human Tendon Allografts In Vitro Study

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Objective: Pre-implantation sterilization procedures for tendons are important measures to reduce the risk of disease transmission, however these procedures may compromise tendon microarchitecture and biomechanical properties to varying degrees. We explore the effects of different sterilization procedures on the micro-histology, biomechanical strength and biochemical properties of human tendon allografts in vitro study.

Methods: The tendon allografts were harvested from cadaveric donors after the donors were serologically screened by antibody or nucleic acid testing of infectious agents. All samples were divided into five groups, which were fresh-frozen group (control group), 15 kGy gamma irradiation group, 25 kGy gamma irradiation group, 70% ethanol group, and peracetic acid-ethanol group. Each group included 10 tendons for testing. Histological staining and transmission electron microscopy were applied to observe the internal structure and arrangement of tendon collagen fibers, while the machine learning classifier was trained to distinguish the darker cross-sections of collagen fibers and brighter backgrounds of the electron micrograph to detect the distribution of diameters of tendon collagen fibers. The visco-elasticity, mechanical properties and material properties of tendon allografts were examined to detect the influence of different intervention factors on the biomechanical properties of tendons.

Results: Histological staining and transmission electron microscopy showed that the structure of fresh-frozen tendons was similar to the structures of other experimental groups, and no obvious fiber disorder or delamination was observed. In the uniaxial cyclic test, the cyclic creep of 25 kGy irradiation group (1.5%) and peracetic acid-ethanol group (1.5%) were significantly lower than that of the control group (3.6%, $F = 1.52$, $P = 0.039$) while in the load-to-failure test, the maximum elongation and maximum strain of the peracetic acid-ethanol group were significantly higher than those of the control group ($F = 4.60$, $P = 0.010$), and there was no significant difference in other biomechanical indicators. According to the experimental results of denatured collagen, it could be seen that no matter which disinfection procedure was used, the denaturation of the tendon sample would be promoted ($F = 1.97$, $P = 0.186$), and high-dose irradiation seemed to cause more damage to collagen fibers than the other two disinfection procedures (296.2 vs 171.1 vs 212.9 $\mu\text{g/g}$).

Conclusion: Biomechanical experiments and collagen denaturation tests showed that 15 kGy gamma irradiation and 70% ethanol can preserve the biomechanical strength and biochemical properties of tendons to the greatest extent, and these two sterilization methods are worthy of further promotion.

Key words: Biochemistry; Biomechanical property; Gamma irradiation; Histology; Peracetic acid; Tendon allograft

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Introduction

As with other forms of allograft, there is a potential risk of spreading bacteria or virus from a donor to a recipient or from contamination of the surrounding environment during the transport and transfer of the allografts. Although serological screening and medical history evaluation have made every effort to reduce the potential risk of disease transmission caused by transplantation of tendon allografts, unfortunately, the risk of disease transmission can never be completely eliminated. Tendon allografts originate from donors who are only allowed to undergo a set of serological screenings, which leaves the possibility of disease transmission for recently infected donors. That is to say, although the recently infected donor has a negative result in the necessary infectious disease test, it is actually infectious in the “window period.”¹

Historically, there are usually two methods for sterilization of tendon allografts: chemical treatment using ethylene oxide gas and radiation sterilization.^{2,3} Although these two sterilization schemes have been proven to be effective sterilization procedures, many scholars had paid close attention to their potential damage to the important physical and biochemical properties of the tendon. Ethylene oxide sterilization is related to causing inflammation in the body,⁴ and the probability of a poor prognosis in patients is greatly increased,⁵ which will eventually lead to the failure of the transplantation operation. Radiation, at a dose sufficient to ensure sterilization effect, has been shown to reduce the mechanical strength of the tendon allografts.⁶ These concerns have led many surgeons to find other safe and effective disinfection techniques.

Ethanol can effectively disinfect allograft bone contaminated by bacteria without destroying the osteogenesis potential, and has been proven in the literature,⁷ and the time and concentration required to sterilize tendon allografts with ethanol have also been explored.⁸ Some scholars have studied the feasibility of using peracetic acid to disinfect tendon allografts.¹ Peracetic acid has been widely used as a cold sterilization procedure for heat-labile equipment in the food industry, and it has also been used in sterilization procedures for allograft bones and heart valves.^{9,10} Peracetic acid is a strong oxidant, formed by the reaction of acetic acid and hydrogen peroxide, and has the advantage of being naturally decomposed into non-toxic or less toxic reactants.¹

The purposes of the current research are to: (i) explore the effects of different sterilization procedures on the microhistology of tendon allografts; (ii) explore the effects of different sterilization procedures on the biomechanical strength and biochemical properties of tendon allografts; and (iii) finally determine the sterilization procedures in vitro that minimize the damage to tendon allografts.

Materials and Methods

Preparation and Treatment of Samples

The tendon allografts used in current study were from Beijing Wonderful Medical Biomaterials (Beijing, China). These

samples were retrieved from cadaveric donors. Donor's next-of-kin gave permission to use tendon samples for scientific research purposes. The tendon allografts were taken out using an aseptic technique in the autopsy room environment and returned to the tissue processing facility. All tendon allografts were serologically screened by antibody or nucleic acid testing before use. All samples were divided into five groups, which are fresh-frozen group (control group), 15 kGy gamma irradiation group, 25 kGy gamma irradiation group, 70% ethanol sterilization group, and peracetic acid-ethanol sterilization group (Fig. 1). Each group included 10 tendons for testing. The sterilized tendons were stored at -80°C .

Disinfection Protocol

The samples in gamma irradiation group were exposed to ^{60}Co gamma radiation doses of 15 kGy (low dose) or 25 kGy (high dose). Sterilization of samples in gamma irradiation group via ^{60}Co radiation was accomplished with the packaged sample on dry ice for varying durations of exposure at equivalent dose rates. Low temperature gamma irradiation (-80°C) was performed to minimize tendon damage and diffusion of free radicals. The radiation dose received by the samples was verified using dosimetry to be within 5% of the target dose. The treated tissues were then wrapped in sterile packaging bags and frozen at -80°C until the time of testing.

Starting from clinical practice, 75% ethanol (China National Pharmaceutical Group Co., Ltd., Beijing, China) was used to disinfect tendon allografts, which was prepared with absolute ethanol and distilled water. Then the tendon allografts were put into the solution and incubated for 2 h. After incubation, the allografts were rinsed in phosphate buffered saline (3×10 min washes).

Peracetic acid (Chengdu Zhongguang Disinfectant Co Ltd., Sichuan, China) was prepared in accordance with the instructions of the specification, and its concentration was determined by iodometry. Anhydrous ethanol and distilled water were used to prepare a peracetic acid-ethanol disinfection solution, the composition of which was 1% peracetic acid and 24% ethanol. Tendon allografts incubated in the peracetic acid solution for 2 h. After the incubation, the allografts were rinsed in phosphate buffered saline (3×10 min washes).

Hematoxylin–Eosin Staining

The mid-substance portions of the tendon samples in experimental group and control group were placed in 10% phosphate-buffered formalin solution (G2161, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for fixation, and then were processed for histological analysis. The samples were embedded in paraffin and microtomed to obtain $5 \mu\text{m}$ -thick longitudinal sections. The sections were attached to glass slides, stained with hematoxylin–eosin (H9627, Sigma-Aldrich, London, UK), and observed under BX53 optical microscopy and photomicrographs were taken (Olympus, Tokyo, Japan).

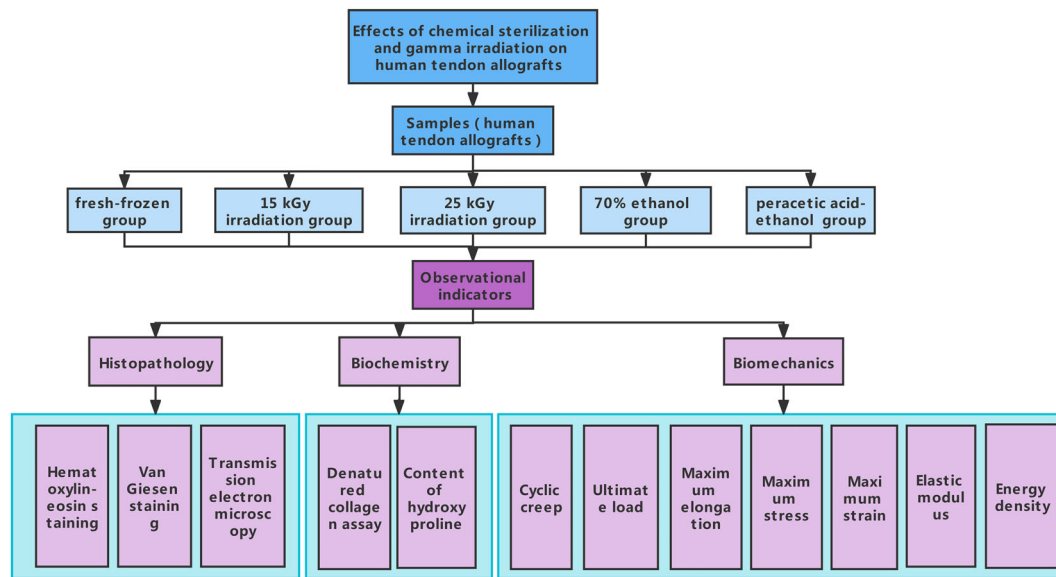


Fig. 1 Experimental flowchart detailing the grouping of the human tendon allografts

Van Gieson Staining

The mid-substance portions of the tendon samples in experimental group and control group were placed in 10% phosphate-buffered formalin solution (G2161, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature for 4 h, and then were processed for histological analysis. The samples were embedded in paraffin and microtomed to obtain 5 μm -thick longitudinal sections. The sections were attached to glass slides, stained with Van Gieson's trichrome (F8129, Sigma-Aldrich, London, UK), and observed under BX53 optical microscopy and photomicrographs were taken (Olympus, Tokyo, Japan).

Transmission Electron Microscopy

Tendon samples for the electron microscopic observation were fixed with 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight (P1126, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). After subsequent buffer washes, the tendon samples were post-fixed in 2.0% osmium tetroxide for 1 h at room temperature and rinsed in DH_2O before staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the sections were stained with uranyl acetate (P1127, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and lead citrate and observed with an electron microscope fitted with a digital camera (H7650, Hitachi High-tech Ltd., Tokyo, Japan).

Cross-sectional micrographs were obtained at 15,000 \times and 30,000 \times magnifications and each electron micrograph was analyzed using a semi-automated protocol with ImageJ/Fiji software (National Institutes of Health, Bethesda, MD, USA). A machine learning classifier was trained to distinguish the darker cross-section of collagen fibers from the

brighter background of the electron micrograph. The collagen fibrils were then selected from the background using the binary thresholding algorithm and separated from the edges of other fibrils using the watershed segmentation algorithm.¹¹ The distribution of fiber diameters was fitted using the Feret's diameter mode that came with the software.

Biomechanical Testing

The mechanical properties of the human tendon samples were assessed using a digital universal testing machine (WDW-10, Jinan Chuanbai Instrument Co., Ltd., Jinan, China). The original cross-sectional area of the tendon sample was measured using aqueous rapid curing alginate dental molding materials and a digital photography and computerized photograph analysis system.¹² This technique marked tendon samples and alginate molds at 1 cm interval and then samples were taken out for mechanical testing. The tendon samples were thawed prior to testing by immersion in a phosphate buffered saline at room temperature and mounted into clamps onto the mechanical testing machine, with the longitudinal axis of the samples aligned vertically and in line with the drawing force. Tendon samples were tested in air at room temperature and saline spray was used to make sure the samples remained moist throughout the tests.

The first test type was uniaxial cyclic test with a cyclic loading between 25 and 80 N for 50 cycles. A preload of 25 N was used to record the initial length of the tendon samples. The range of load was determined based on the results of previous tests and literature to ensure that the tendon could be stretched enough without causing rupture.³ After 50 cycles of loading were over, the displacement-load curve and the final length of the tendon sample were recorded to calculate the cyclic creep.

The second test type was load-to-failure test using a strain rate of 2 mm/s. The load was measured using a load cell and the load and displacement were recorded every 50 MS. Failure was defined as the point at which the tendon sample could no longer sustain the drawing force and the failure modes were defined as mid-substance or avulsion.

The ultimate load, maximum elongation, maximum stress, maximum strain, elastic modulus, and energy density were calculated from the stress-strain curve. The load was converted into axial stress value based on the original cross-sectional area. The strain was based on the displacement of tendon sample and reference length, which defined as the total distance between the clamps when the preload was applied. Elastic modulus, a measurement of the sample stiffness, was defined as the tangent of the stress-strain curve at the point of maximum steepness.

Collagenase Susceptibility

The tendon samples were blotted dry and weighed. Tendon samples were incubated in 3 ml of 0.1% (w/v) α -chymotrypsin pH 7.8 in Tris-HCl buffer with 50 mM calcium chloride for 24 h at 37°C with gentle agitation (C8660, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). After 24 h of incubation, all samples of the digest were taken and centrifuged at 13,000 g for 10 min to pellet any particulate matter. The assay used for testing the amount of hydroxyproline was a modification of that of Reddy and Enwemeka¹⁵ and related kits were used (BC0250, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China).

A series of hydroxyproline standard solutions, ranging from 0.117 to 15 μ g/ml were prepared and assayed as below. Samples of the supernatant (200 μ l) were pipetted into sealable 2 ml polypropylene centrifuge tubes. Following pipetting, 200 μ l of the first reagent was added to each tube, and the tubes incubated at ambient temperature for 20 min. Following this incubation, 200 μ l of the second reagent was added to each tube, and these tubes placed in a 60°C water bath for 20 min. Duplicate aliquots (1 ml) of all tendon and standard samples were then transferred to cuvettes and absorbance read at 560 nm, against the reagent blank. The experimental results were used to assess the amount of hydroxyproline removed per gram of tendon sample.

Observational Indicators

Gaps between Tendon Fibers

The gap between collagen fibers refers to the blank area between collagen fibers without cells and extracellular matrix. The histology of tendon sample was evaluated and observed by Hematoxylin-eosin staining and Van Giesen staining. In Hematoxylin-eosin staining, collagen fibers and extracellular matrix components were stained pink, and cell nuclei were stained blue; in Van Giesen staining, collagen fibers were stained red, and deformed collagen fibers were stained yellow. Using an ordinary optical microscope, we could observe whether the tendon fibers were disordered,

broken, or bent under various intervention factors, and then judge the adverse effects of the intervention factors on the overall tendon samples.

Distribution of Fiber Diameters

Tendon is a kind of fibrous connective tissue, and its structural size ranges from a few nanometers to a few millimeters. The thickness of collagen fibers in the tendon refers to the distribution of fiber diameters. In order to observe the tendon components of different scales, transmission electron microscopy is often performed.

To quantitatively analyze the breadth and symmetry of distribution of tendon fiber diameter, fiber diameters can be divided into four groups: 0–50, 50–100, 100–150, and 150–200 nm.¹⁴ A machine learning classifier was trained to distinguish the darker cross-section of collagen fibers from the brighter background of the electron micrograph.

Viscoelasticity

The creep of tendon means that the deformation of the tendon increases with time under constant stress. The viscoelasticity of tendon sample was quantified by measuring the cyclic creep. If the viscoelasticity of the tendon becomes significantly lower, it indicates that it is difficult to ensure sufficient deformation when the tendon is pulled by an external force, which may result in rupture of the tendon.

Mechanical Properties

The mechanical properties of the tendon samples included ultimate load and maximum elongation in the load-to-failure test. If the ultimate load and maximum elongation of the tendon are significantly lower, it indicates that the tendon will be more likely to rupture and damage when the tendon is subjected to an external force exceeding the critical point.

Material Properties in Biomechanics Test

The material properties of the tendon included maximum stress, maximum strain, elastic modulus, and energy density in the load-to-failure test.

Compared with the ultimate load, the maximum stress excludes the influence of the cross-sectional area on the biomechanical data to the greatest extent, and can more objectively reflect the biological strength of the tendon sample. If the maximum stress of the tendon is reduced, the tendon is more likely to break when it is stretched by an external force.

Maximum strain is the relative percentage of the maximum deformation of the tested tendon sample compared to the initial length under stress. Compared with the maximum elongation, the maximum strain is a ratio rather than an absolute difference, so it is more accurate and objective for evaluating the deformation of the tendon.

The elastic modulus can be regarded as an indicator of the difficulty of elastic deformation of the tendon. The greater the value, the greater the stress that causes the tendon to undergo a certain elastic deformation, that is, the greater

the stiffness of the material. If the elastic modulus of the tendon increases, it proves that the elastic deformation of the tendon under the action of external force is more difficult, and the material of the tendon itself is harder.

Energy density refers to the area under the stress-strain curve, which represents the energy required to cause the structure of the tendon to be destroyed, also known as toughness.

The Content of Hydroxyproline

After the tendon is hydrolyzed by collagenase, a certain amount of hydroxyproline will be released. The stability of the tendon can be tested by determining the content of hydroxyproline. To assess the potential changes in the integrity of collagen fibers, an assay measuring denatured collagen was applied. This assay had been used in testing of tissues including dermis, bone, tendon, and cartilage.¹⁵⁻¹⁷ If the content of hydroxyproline detected in the digest solution of a certain group of tendon is significantly higher than that of the control group, it means that the corresponding intervention factor has largely destroyed the triple-helical structure of collagen, thereby making the tendons tend to be unstable and subject to denaturation.

Statistical Analysis

Normally distributed continuous variables were presented as mean \pm standard deviation (cross-sectional area, creep, ultimate load, maximum elongation, maximum stress, maximum strain, elastic modulus, energy density, content of hydroxyproline), and categorical variables were expressed as proportions (fiber diameter distribution). All data were firstly analyzed by one-way analysis of variance, and then Kruskal-Wallis test was used to analyze the data that did not conform to the homogeneity of variance and the normal distribution. Statistical analysis was performed using R version 3.5.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 8 Software (GraphPad Software Inc., San Diego, CA), and *P* value <0.05 (two-sided) was considered statistically significant.

Results

Histological Staining

The histology of the tendons after different sterilization treatments was shown in Figs 2 and 3. The integrity of the tendon structure remained basically unchanged after sterilization by different methods. The tendons in the control group maintained the same direction and consistent shape, with no obvious fiber breakage and disorder, and the interstitial space was relatively tight. Compared with the control group, the interstitial space of the tendon in the 15 kGy gamma irradiation group was enlarged, but the collagen fibers still maintained the same direction and shape; and there was no obvious rupture. In the 25 kGy gamma irradiation group, the interstitial space of the tendon was further expanded, and the collagen fibers were curled and

disordered; the fiber orientation was inconsistent. The gaps between the collagen fibers of the tendon in the 70% ethanol group were widened and the curled waveform was slightly loosened, and the crimp waveforms were loosened slightly. Similar to the 70% ethanol group, the gaps between collagen fibers in the peracetic acid-ethanol group were widened and the crimp waveforms loosened slightly, and relatively more nuclei were visible.

Distribution of Fiber Diameters

Transmission electron microscopy showed that the structure of fresh-frozen tendons was similar to the structures of other experimental groups, and no obvious fiber disorder or delamination was observed (Fig. 4). The following analysis of fiber diameter distribution provided further quantitative analysis of the results (Fig. 5A). Through the identification of image analysis software, it could be known that the fiber diameters of the control group mostly distributed in the range of less than 100 nm (84.6%); while the fiber diameters of the other groups evenly distributed in various stages of 0–200 nm, that is, the proportion of large-diameter fibers (100–200 nm) had been significantly increased in these groups (36.6%–53.7%). It is worth noting that the proportion of fiber diameter >150 nm in the 25 kGy gamma irradiation group was less (6.1%), which was similar to that of fresh frozen tendon (6.8%). This may be one of the reasons that the macroscopic diameter of the tendon samples in the 25 kGy irradiation group was smaller than that of 15 kGy irradiation group (11.82 vs 12.88 mm², *F* = 3.40, *p* = 0.031, Fig. 5B).

Viscoelasticity

In the uniaxial cyclic test, the cyclic creep of five groups of tendon samples were significantly different (*F* = 1.52, *P* = 0.039). Specifically, the cyclic creep of 25 kGy irradiation group (1.5%) and peracetic acid-ethanol group (1.5%) were significantly lower than that of the fresh-frozen group (3.6%). There was no statistically significant difference between the values of 15 kGy irradiation group (3.9%) and 70% ethanol group (2.6%) compared with the fresh-frozen group (Table 1; Fig. 6A).

Mechanical Properties

For the ultimate load, the value of 25 kGy irradiation group (392.9 N) was significantly lower than that of the control group (513.9 N), and the values of the remaining groups were higher than that of the control group, although there was no statistically significant difference in the data between the five groups (*F* = 2.11, *P* = 0.124, Table 1).

For the maximum elongation, there was a statistically significant difference in the data between the five groups (*F* = 4.59, *P* = 0.010), specifically, the maximum elongation of peracetic acid-ethanol group (6.5 mm) was significantly greater than the value of the control group (4.0 mm). There was no significant difference in the maximum elongation of the remaining four groups compared with the control group (Table 1).

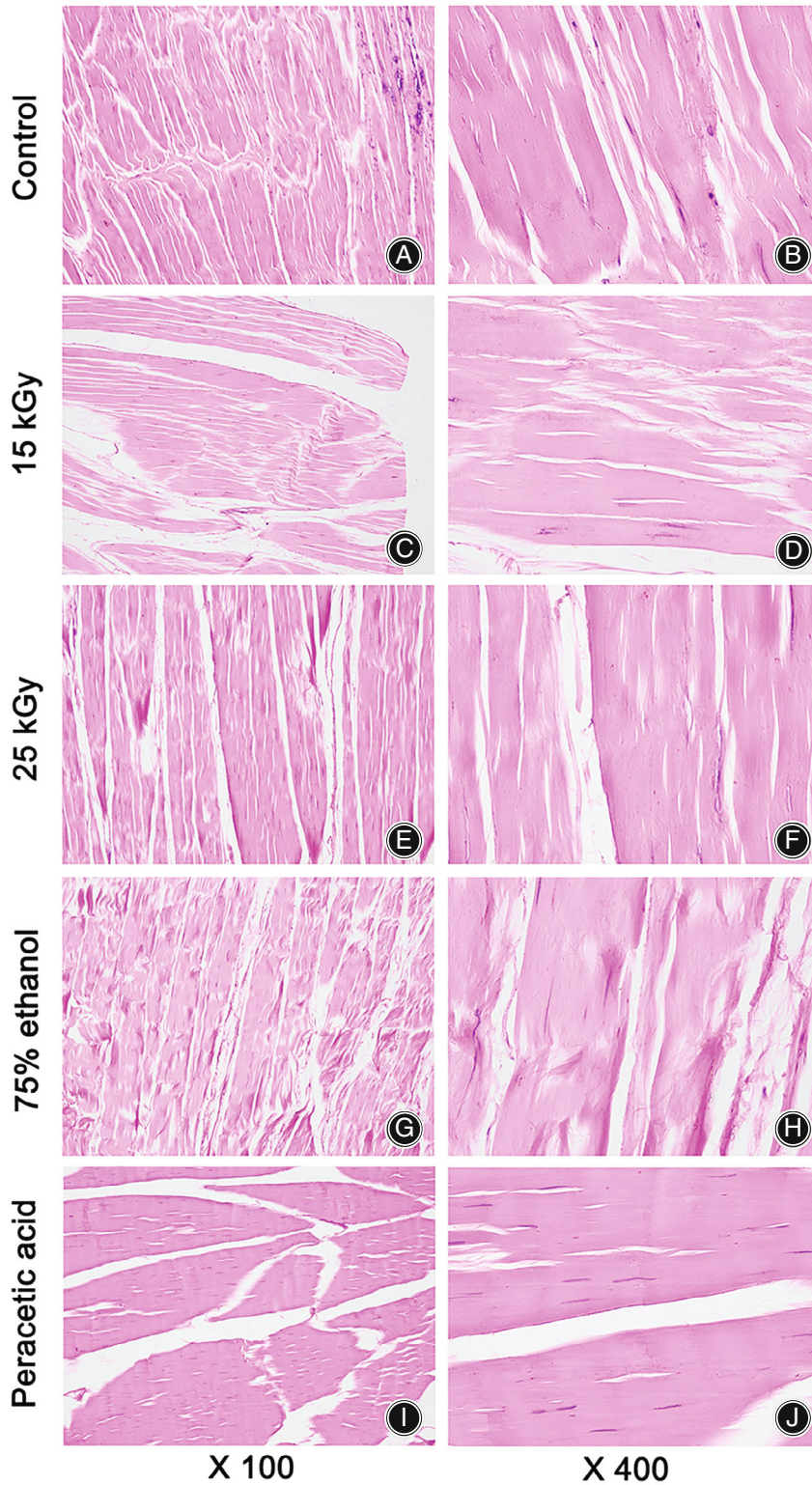


Fig. 2 Histology of tendon allografts with different treatments using Hematoxylin–eosin staining. Representative micro images of internal structure of the fresh-frozen group (A and B), 15 kGy gamma irradiation group (C and D), 25 kGy gamma irradiation group (E and F), 70% ethanol sterilization group (G and H), peracetic acid-ethanol sterilization group (I and J), taken by the optical microscopy at 100 \times (left panels) and 400 \times (right panels), respectively

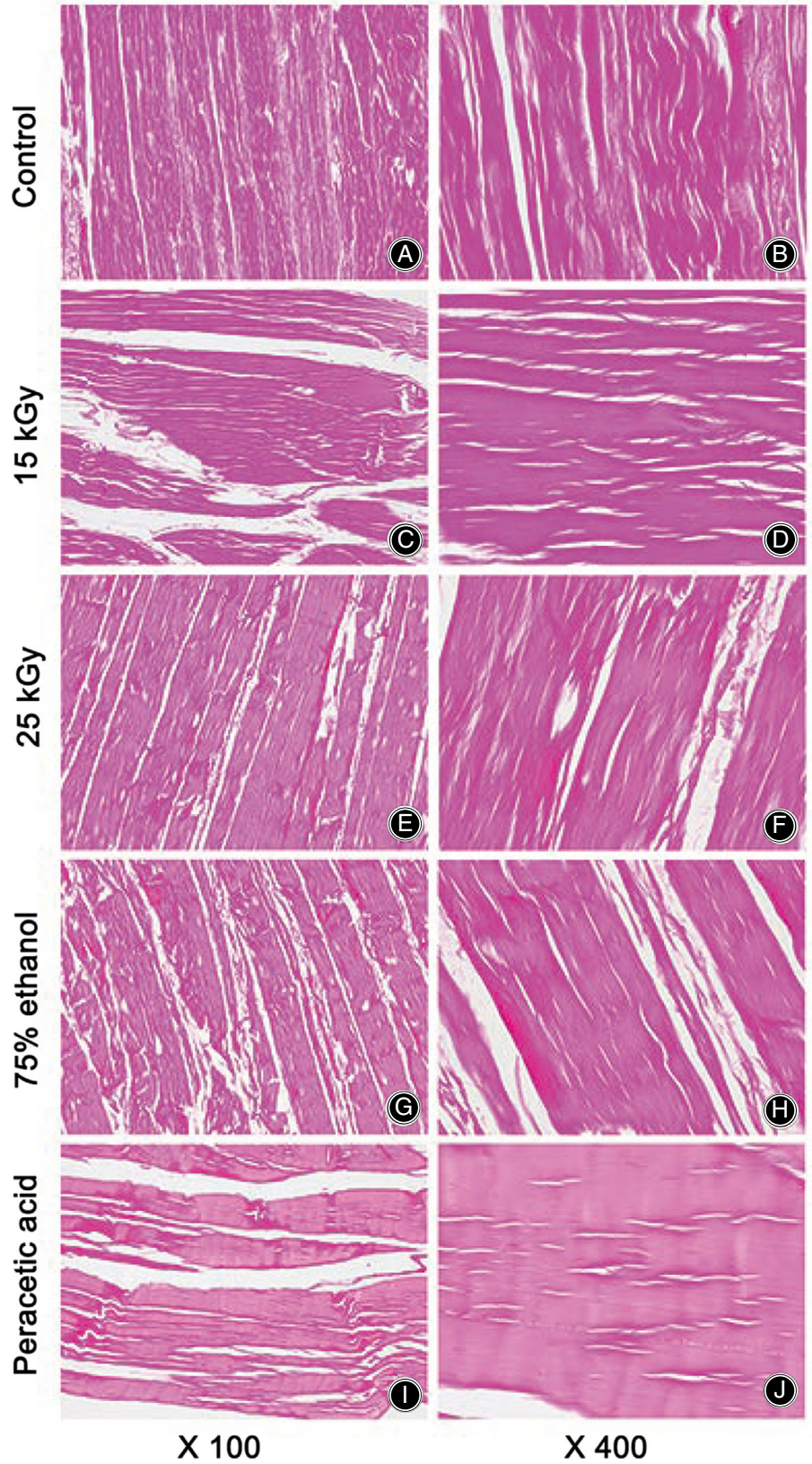


Fig. 3 Histology of tendon allografts with different treatments using Van Gieson staining. Representative micro images of internal structure of the fresh-frozen group (A and B), 15 kGy gamma irradiation group (C and D), 25 kGy gamma irradiation group (E and F), 70% ethanol sterilization group (G and H), peracetic acid-ethanol sterilization group (I and J), taken by the optical microscopy at 100 \times (left panels) and 400 \times (right panels), respectively

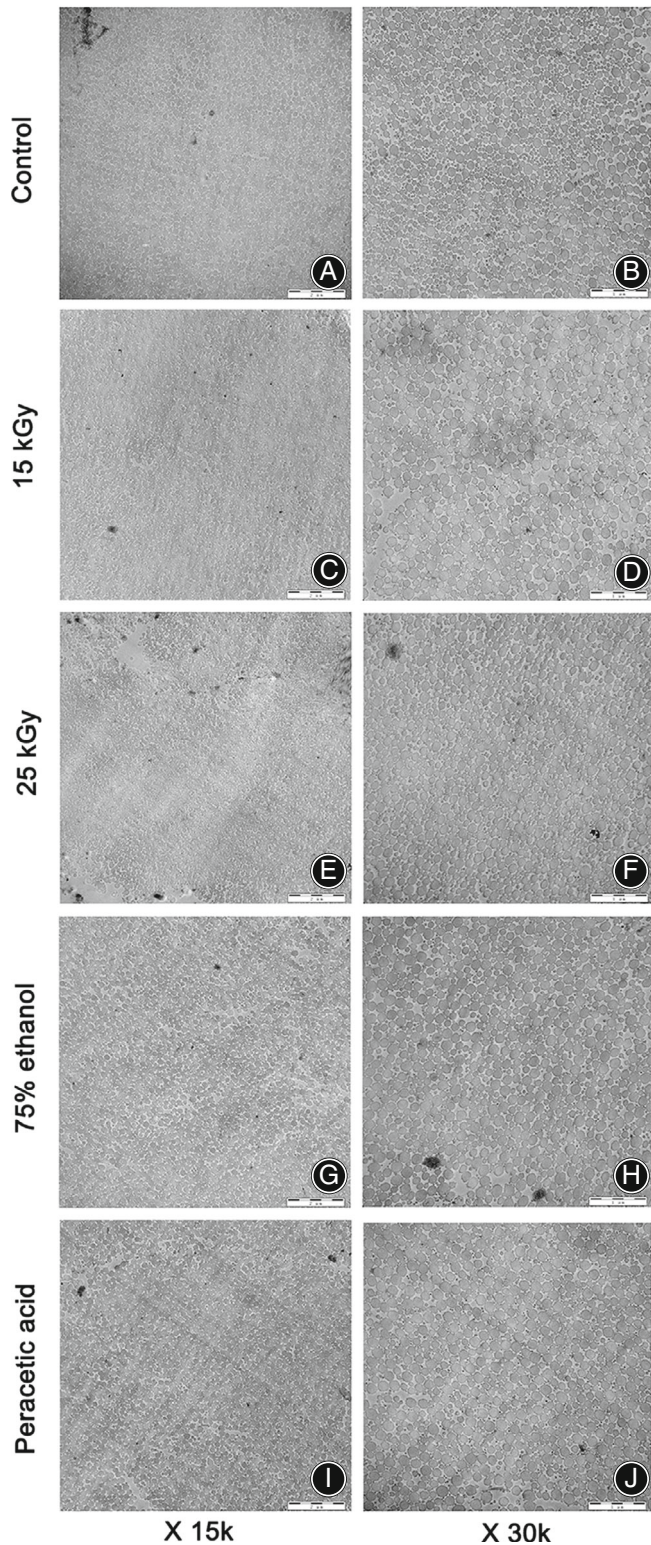


Fig. 4 Representative micro images of cross-section of tendon samples in fresh-frozen group (A and B), 15 kGy gamma irradiation group (C and D), 25 kGy gamma irradiation group (E and F), 70% ethanol sterilization group (G and H), peracetic acid-ethanol sterilization group (I and J), taken by the electron microscope at 15,000 \times (left panels) and 30,000 \times (right panels), respectively

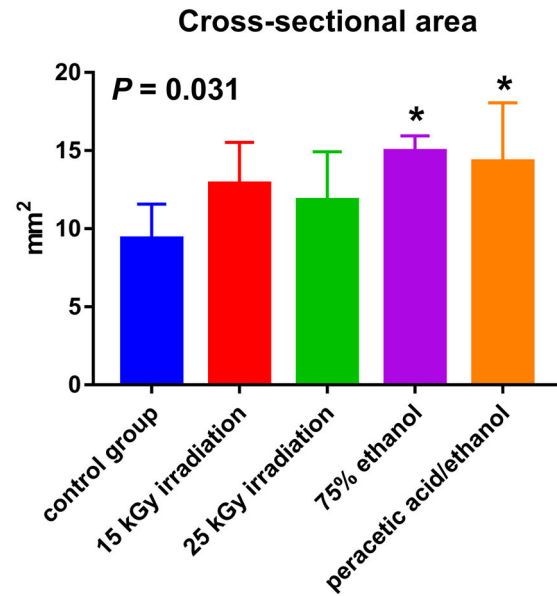
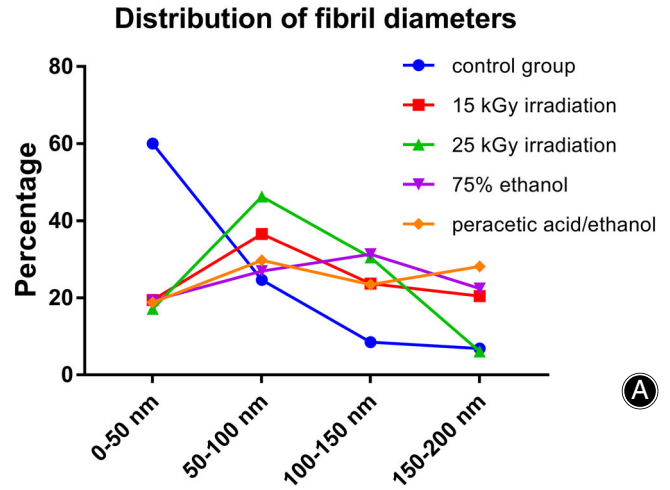


Fig. 5 The distribution of fibril diameter (A) and the cross-sectional area (B) of tendons in each group of samples. After the sterilization procedures, the fibril diameter and cross-sectional area of each group increased to varying degrees

Material Properties in Biomechanics Test

For the maximum stress, the performances of five groups of tendon samples were similar ($F = 2.58$, $P = 0.075$). Although the value of 25 kGy irradiation group (33.4 Mpa) seemed to be significantly lower than the value of the control group (54.2 Mpa), this conclusion was not supported by statistical tests (Table 1; Fig. 6B).

For the maximum strain, there were significant differences in the performances of the five groups of tendon samples ($F = 4.60$, $P = 0.010$). Similar to the results of the maximum elongation, the value of peracetic acid-ethanol group (13.0%) was significantly higher than that of the

TABLE 1 The effects of different treatments on the biomechanical properties of tendon allografts

Group	Cross-sectional area (mm ²)	Creep (%)	Ultimate load (N)	Maximum elongation (mm)	Maximum stress (Mpa)	Maximum strain (%)	Elastic modulus (Mpa)	Energy density (kJ·m ⁻²)
Fresh-frozen group	9.4 ± 2.2	3.6 ± 2.5	513.9 ± 164.1	4.0 ± 1.0	54.2 ± 7.1	8.1 ± 2.1	878.0 ± 217.1	3.2 ± 1.3
15 kGy irradiation	12.8 ± 2.6	3.9 ± 3.8	717.8 ± 311.1	4.9 ± 1.2	53.9 ± 12.1	9.8 ± 2.4	759.1 ± 142.9	3.6 ± 1.5
25 kGy irradiation	11.8 ± 3.1	1.5 ± 0.4	392.9 ± 257.2	4.8 ± 1.1	33.4 ± 12.4	9.7 ± 2.2	523.9 ± 250.3	2.9 ± 1.1
75% ethanol	14.9 ± 0.9	2.6 ± 0.4	810.4 ± 239.4	4.4 ± 0.8	53.9 ± 14.7	8.9 ± 1.7	595.5 ± 116.8	3.4 ± 0.4
Peracetic acid-ethanol	14.3 ± 3.7	1.5 ± 0.6	628.7 ± 219.7	6.5 ± 0.6	44.1 ± 11.2	13.0 ± 1.2	778.2 ± 219.9	5.0 ± 1.8
Overall	12.5 ± 3.2	2.6 ± 2.1	617.2 ± 263.0	4.9 ± 1.2	48.5 ± 13.4	9.9 ± 2.5	711.8 ± 216.7	3.6 ± 1.4
F value/H value	3.403	10.100	2.111	4.597	2.576	4.602	2.568	1.623
p value	0.031	0.039	0.124	0.010	0.075	0.010	0.073	0.212

Note: Normally distributed continuous variables were presented as mean ± standard deviation.

control group (8.1%), and the values of the remaining four groups remained basically the same as the control group (Table 1; Fig. 6C).

In terms of elastic modulus and energy density, no significant differences between different intervention factors were found ($F = 2.57$, $P = 0.073$; $F = 1.62$, $P = 0.212$). After undergoing the disinfection procedures, the elastic modulus of each group of tendon samples had decreased to varying degrees compared with the control group; however, the trend of changes in energy density was a state of coexistence of increasing and decreasing (Table 1; Fig. 6D).

The Content of Hydroxyproline

The results of the denatured collagen assay was shown in Fig. 7. After digestion with α -chymotrypsin for 24 h, >100 μ g denatured collagen was observed in any group, as determined by release of hydroxyproline per gram of sample tissue, and there was no significant difference between experimental groups and the control group ($F = 1.97$, $P = 0.186$). According to the experimental results, it could be seen that no matter which disinfection procedure was used, the denaturation of the tendon sample would be promoted, and irradiation (no matter low-dose or high-dose) seemed to cause more damage to collagen fibers than the other two disinfection procedures (204.6 and 296.2 μ g/g vs 171.1 and 212.9 μ g/g).

Discussion

The current study investigated the effects of several commonly used sterilization methods on the histology, biomechanics and biochemistry of human tendon allografts. Histological staining and transmission electron microscopy showed that the structure of fresh-frozen tendons was similar to the structures of other experimental groups, and no obvious fiber disorder or delamination was observed. In the uniaxial cyclic test, the cyclic creep of 25 kGy irradiation group and peracetic acid-ethanol group were significantly lower than that of the control group, while in the load-to-failure test, the maximum elongation and maximum strain of the

peracetic acid-ethanol group were significantly higher than those of the control group, and there was no significant difference in other biomechanical indicators. According to the experimental results of denatured collagen, it could be seen that no matter which disinfection procedure was used, the denaturation of the tendon sample would be promoted, and high-dose irradiation seemed to cause more damage to collagen fibers than the other two disinfection procedures.

Severe tendon avulsion usually requires tendon autograft or allograft to reconstruct the damaged tissue.¹⁸ Although tendon autograft has the best biocompatibility, there remain many problems such as donor-site complications and secondary injury.¹⁹ To solve the shortcomings of tendon autografts, some synthetic grafts and tissue engineered tendons had been developed, but further in vivo and in vitro experiments and clinical trials are needed to verify the effectiveness and safety before clinical application.^{20,21}

Tendon allograft seems to be an ideal alternate graft. However, some scholars worry about whether the implanted graft is free of bacterial, viral or fungal contamination, because adverse events had been reported.^{22,23} To minimize the possibility of disease transmission, several processing and disinfection procedures have been proposed. In addition to being effective in killing a variety of pathogens, the disinfection procedure should also retain the normal inherent properties and performance of the tendon itself.

Distribution of Fiber Diameters

Histological analysis and transmission electron microscopic observation showed that after the tendon sample had undergone disinfection procedures, the tendon fibers became swollen, and the fiber diameter and interfibre space also became larger, regardless of the type of disinfection procedure. There is no doubt that the increase in spaces between collagen fibers will affect the biomechanical strength of the tendon, but it may also promote cell migration and nutrient penetration. Huang *et al.* evaluated the effect of peracetic acid treatment and cell removal procedure on tendon matrix generation. The results showed that the integrity of tendon

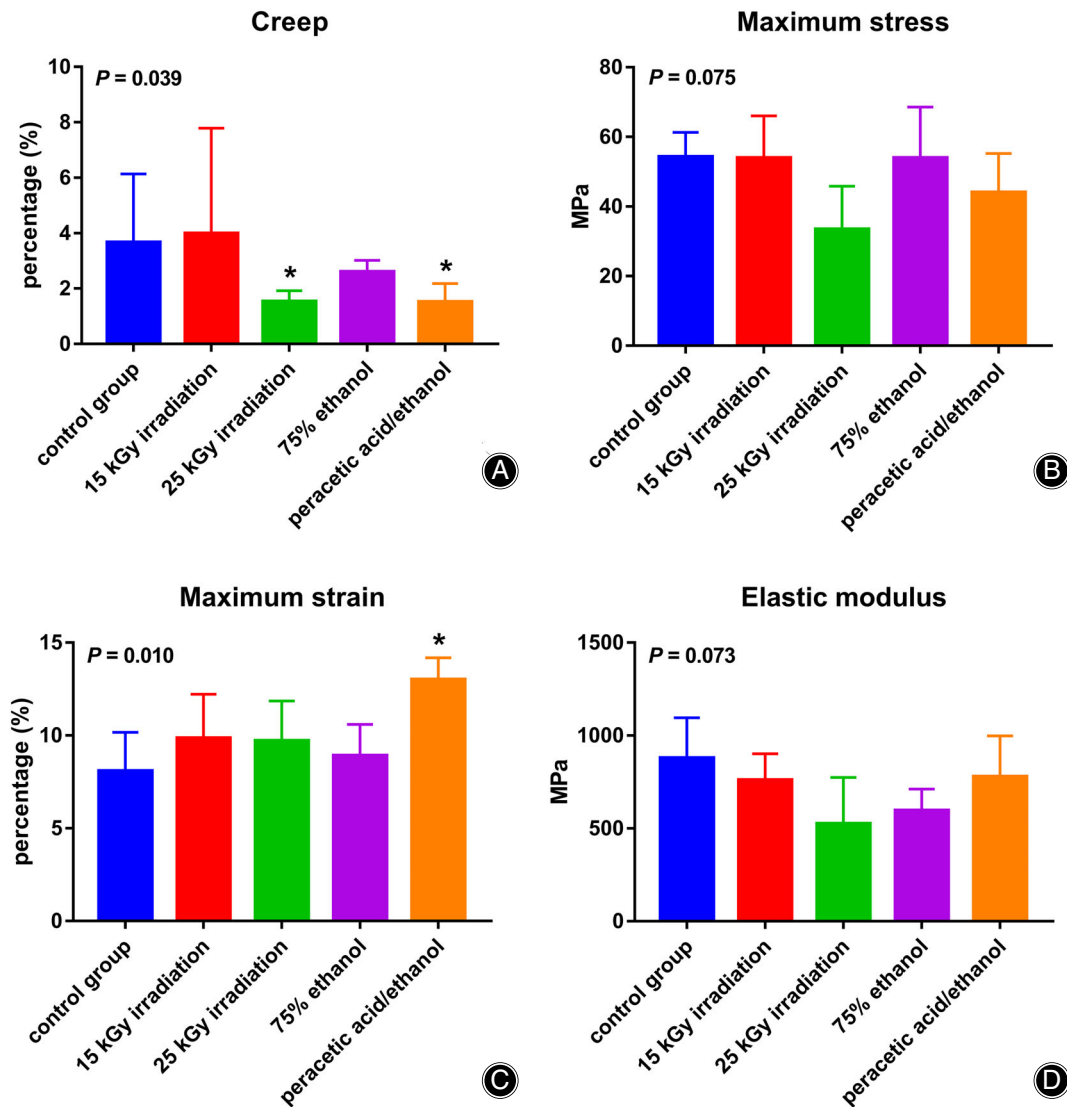


Fig. 6 Comparison of the biomechanical indicators of each group of samples, including creep (A), maximum stress (B), maximum strain (C), and elastic modulus (D). Significant difference between groups were shown in terms of cyclic creep ($P = 0.039$) and maximum strain ($P = 0.010$)

structure was maintained after decellularization and peracetic acid sterilization, but the collagen waveform was slightly loosened. They point out that increased space between collagen fibers may promote cell proliferation and differentiation, and may ultimately promote tendon healing.²⁴

The spaces between the fibers in the peracetic acid-ethanol group may be caused by the oxidation of peracetic acid. The oxidation of peracetic acid can inactivate potential viruses and bacteria while increasing the porosity of the tendon.³ At the same time, through the penetration of ethanol, tendon fibers may also become swollen.

When gamma rays were used to irradiate tendons, the energy directly released by the gamma rays and the accompanying free radicals would induce the degeneration and destruction of the collagen fibers and other structures in

tendons, which might cause the tendon fibers to appear disordered and increase the interfibre space.^{25,26} Schwartz *et al.*²⁶ explored the effects of gamma irradiation on the biomechanical and biochemical properties of anterior cruciate ligament allografts in a goat model. The authors performed bilateral anterior cruciate ligament reconstruction in 18 adult goats, one knee receiving an irradiated patellar tendon allograft (4 Mrad) and the other receiving a frozen allograft (0 Mrad). By 6 months, irradiated grafts had lower stiffness and maximal force compared to control group, but no differences in modulus, maximal stress, or biochemistry. The authors therefore argue that while high levels of gamma irradiation may inactivate infectious agents, this treatment is not a viable clinical option due to altered allograft biomechanics. However, what is surprising is that histological analysis and

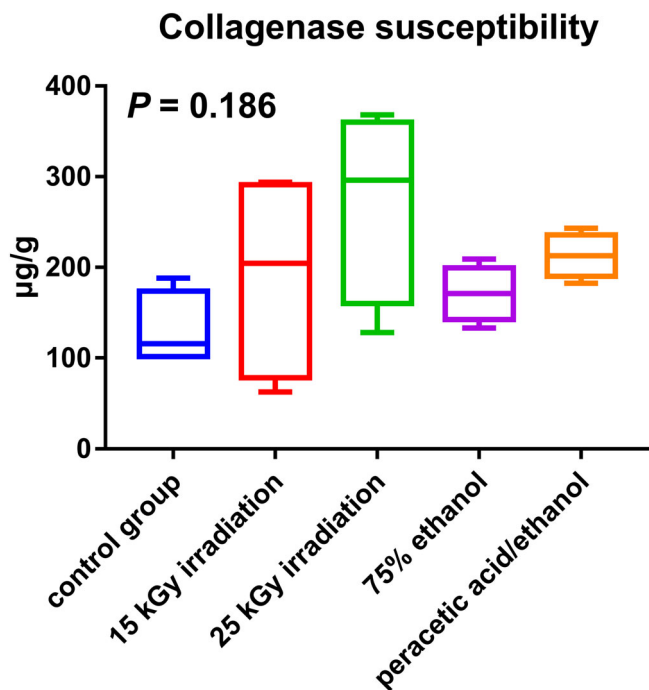


Fig. 7 Measurement of soluble collagen in the digest solution as a proportion of tendon weight after incubation with α -chymotrypsin. It could be seen that irradiation (no matter low-dose or high-dose) seemed to cause more damage to collagen fibers than the other two disinfection procedures

transmission electron microscopy did not find a dose-response relationship between radiation dose and tissue damage, and even a higher dose of radiation was accompanied by a smaller interfibre space. This may be due to the small difference between these dose gradients in our study.

Mechanical and Material Properties

Most of the biomechanical indicators showed no significant difference between the control group and the experimental groups, except for cyclic creep and maximum strain. The cyclic creep of 25 kGy irradiation group and peracetic acid-ethanol group were significantly lower than that of the control group. As mentioned earlier, the deformation of the tendon has a significant energy storage effect. For harmful external forces, the tendon can absorb harmful energy in the form of deformation to avoid further damage. Regarding the maximum elongation and maximum strain, the peracetic acid-ethanol group showed much higher values than other groups, which means that peracetic acid has a greater influence on the extension of the collagen fibers.

In an *in vitro* experiment conducted by Lomas and colleagues, the effect of peracetic acid on the biomechanical properties of tendon was limited to the increase in ultimate strain.¹ The extension or strain of the tendon under load

may be related to the extension of the collagen fibers, or the orientation, and relative sliding of the fibers relative to each other. The latter is more likely to lead to increased strain. Similar results were also observed by Scheffler and colleagues. In an *in vitro* biomechanical study, Scheffler *et al.*²⁷ investigated whether peracetic acid ethanol sterilization had any adverse effect on the mechanical properties of human bone-patellar tendon-bone grafts. It was found that the strain of the tendon after sterilization with peracetic acid ethanol increased, but the cyclic creep showed a decreasing trend. This suggests that peracetic acid ethanol may have some effect on the elastic components of the graft, such as collagen fibers and connective tissue.

The Content of Hydroxyproline

To study the changes in the integrity of collagen, we used the assay of denatured collagen. The results showed that the content of denatured collagen in the two irradiated groups was higher than that in the other groups, and the effect was more pronounced in the higher-dose group, although this difference had not yet reached statistical significance. One possible explanation is that higher dose of irradiation will cause the breakage of covalent bonds such as peptide bonds and intermolecular hydrogen bonds within collagen molecules, and at the same time destroy mature molecular crosslinks and induce the formation of immature intermolecular crosslinks. As a result, the internal structure of the tendon is destroyed.²⁸

Huang *et al.*²⁴ evaluated the effects of peracetic acid and cell removal procedures on the biochemical properties of tendons using 0.1% (w/v) peracetic acid for 3 h. The results showed that the measurement of denatured collagen in the experimental group showed no significant increase compared to the control. This indicates that the decellularization and sterilization treatment procedures did not impair the main properties of the tendon. This is consistent with our findings that ethanol and peracetic acid have no effect on the biochemical properties of tendons.

Limitations

The current research has several limitations. First, no cytotoxicity test or biocompatibility test were performed, which is very important because tendon allograft will be implanted into the body. Further *in vivo* or *in vitro* studies should be designed to determine the biocompatibility of tendon allografts. Second, the human tendons used in this study did not restrict one specific sampling site, which led to some differences between groups that may not be caused by the intervention factors themselves, but due to the differences in the sampling sites. In future research, the record and standardization of the sampling sites should be further refined. Finally, we only explored the effect of gamma irradiation at two doses of 15 and 25 kGy on the tendon, and there is no more subdivided dose parameters. This shortcoming is hoped to be addressed in future studies.

Conclusions

We described and evaluated the effects of different disinfection procedures on tendon allografts. Histological evaluation showed that both irradiation and chemical disinfection procedures would enlarge the gaps between collagen fibers. Biomechanical experiments and collagen denaturation tests showed that 15 kGy gamma irradiation and 70% ethanol can

preserve the biomechanical strength and biochemical properties of tendon to the greatest extent, and these two sterilization methods are worthy of further promotion. In order to explore the impact of disinfection procedures on biocompatibility and tendon-bone healing, further *in vivo* and *in vitro* experiments are necessary.

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