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Evaluation of the bovine tendon decellularization method in the development of a cruciate ligament prosthesis

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Abstract

Introduction. *Ligaments play a critical role in the body, linking bones together. Ligament ruptures are the most common manifestations of serious musculoskeletal injuries. This is especially true for the anterior cruciate ligament of the knee joint. In trauma surgery, orthopedics, the ligament functional recovery can be achieved by using tissue autografting, synthetic and biological prostheses. Of great interest to surgeons is the use of prostheses made of biological tissues of animal origin, since they are easily available and, when high-quality processing is achieved, they are safe, retaining their natural structure and strength.*

Purpose of the study. *To decellularize the bovine tendon according to our originally developed technique and to investigate its efficacy in terms of the presence of cellular elements and physical and mechanical parameters of the material.*

Material and methods. *To manufacture the prototype of the ligament prosthesis product, we developed the technique for processing the bovine tendon, as the most similar material by structure, including its mechanical processing, chemical and physical processing methods and a*

special treatment with supercritical carbon dioxide fluid containing nonionic surfactant Tween-80 for decellularization and extraction of organic components in addition to collagen framework, while maintaining strength properties. Histological studies were performed to check for the residues of cellular elements, and the measurements of the physical and mechanical properties of the material were made.

Results. *Histological examination of the material showed that after processing, 0–2 cells in the field of view were found in the material. The strength properties of the material were 503 kgf/mm^2 before processing and 605 kgf/mm^2 after processing.*

Conclusion. *The data obtained in the study confirmed that the processing performed qualitatively affected the elimination of cells, did not worsen, but even increased the mechanical strength of the material. Further study of the biocompatible properties of the material is required.*

Keywords: ligament prosthesis, decellularization, tendon, xenograft, biocompatibility, supercritical carbon dioxide fluid

Conflict of interests Authors declare no conflict of interest

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ACL, anterior cruciate ligament

SCF-CO_2, supercritical carbon dioxide fluid

Introduction

Ligaments play an essential role in the body, connecting bones together. They are bands of dense connective tissue, predominantly composed of collagen and elastin proteins. The anterior cruciate ligament (ACL) that courses from the femur to the tibia is of greatest importance in trauma surgery. It averages 32 mm long and 7–12 mm wide. The fibers inside the ligament are twisted at an angle of 110°. The ligament is a key structure in the knee joint, as it resists the lower leg shifting forward and inward. Up to 50–70% of all injuries of the musculoskeletal system are injuries of the knee joint; mainly among athletes, representatives of physically heavy sports and martial arts. ACL rupture is one of the most common causes of serious musculoskeletal injury typical of physically active people [1]. Theoretically, the ultimate load on the ACL is in the range from 734 N (73.4 kgf/mm^2) to 1725 N (172.5 kgf/mm^2), and the mean load on the ACL for a human from 17 to 35 years old is 1700 N (170 kgf/mm^2) [2].

It is mostly the people of the best working age who are commonly susceptible to injuries of the knee joint; meanwhile, men are injured on average 2 times more often than women [3]. Technology improvements for reconstruction surgery of the ACL, and other types of knee ligaments or other joints, has been highly relevant for orthopedic trauma surgeons for many decades.

The main reasons for the interest in the problem of ligament rupture were not only the persisting tendencies towards an increase in the number of knee injuries associated with ligament ruptures, the emergence of new methods of reconstructive surgery and its technical equipment, but also the need that emerged in the professional community to determine the advantages, features, and prospects of the technologies being created [4].

In arthroscopic surgery, the issue of choosing a graft for reconstructive surgery remains topical. For this purpose, auto- and allografts, as well as synthetic prostheses have been used. The most suitable of autologous tissues for ligament reconstruction are the medial third of the native patellar ligament with bone graft, tendon, semitendinosus and gracilis muscles as well as a part of the femur quadriceps tendon with a bone graft. Donor tendons preserved by various methods are used as allografts. Synthetic ligament prostheses are made of very durable synthetic materials such as nylon, lavsan, perylene, dacron, and polyester. Disadvantages of autografting include an increased duration and invasiveness of surgery, the presence of a donor site wound and associated complications. Synthetic prostheses, despite their significant advantages such as minimized surgical trauma, early mobilization of patients, the possibility of load and movement immediately after surgery, also have considerable disadvantages: synovitis, progressive osteolysis around the canals in the tibia and femur condyles, a decreased capacity to transformation, and a rapid build-up of degenerative-dystrophic abnormalities in the knee joints. Biological prostheses are free from these disadvantages. However, the use of allo- and xenografts carries a risk of infection transmission; they are characterized by more pronounced osteolysis of the bone canals due to the immune response and a greater decrease in the mechanical properties of the graft, especially in the first 6 months, uncontrolled bioresorption and biodegradation, as well as an increased cost of surgery. These problems are primarily associated with an insufficient removal of the biological components the recipient's body responds to, or with the collagen framework destruction due to the harsh chemical-enzymatic methods used to remove these biocomponents from the tissue [5]. It is worth paying special attention to flexible biomaterials of xenogeneic

origin, which are based on collagen, the natural protein of connective tissue. These biomaterials are highly similar in structure to the body tissues to be replaced and are easily available.

The most promising trend in repairing tissue defects in reconstructive surgery today is the development of bioimplants based on collagen-containing biological tissues such as decellularized xenopericardium [6, 7]. However, for a ligament prosthesis, in particular, the cruciate ligament in the knee, the required starting material should possess very high initial strength characteristics, be capable to withstand, at least, the threshold loads inherent in the ligaments, have similar structure, and be of various lengths and cross-sectional areas for the selection of the necessary size of the prosthetic material by clinicians. Tendons are the most suitable material meeting these criteria. As already mentioned, the main disadvantages of biomaterials, including those of xenogeneic origin, are their ability to induce an inflammation and rejection, as well as infection due to the presence of alien cellular elements and biological components.

When creating xenografts, the priority task and requirement in pre-implantation processing of donor tissue are to reduce the risks of the surrounding tissue inflammation and potential graft rejection. One of the methods for creating tissue implants to be used in reconstructive surgery to improve reparative processes and renew structural and functional elements in damaged tissues and organs includes the devitalization of animal donor tissues. Devitalization solves the following tasks: prevents a rejection by removing or destroying donor cells from the implanted sample (decellularization), stabilizes the tissue structure (preservation), retains adequate biomechanical properties and sterility of the material. Most of the methods for obtaining tissue bioimplants from xenogeneic tissues are based on prolonged processing with various detergent-enzyme

and preservative solutions (glutaraldehyde, epoxy compounds), hypo- and hypertonic solutions, which action is associated with the destruction of biological tissue components and its structural stabilization. These methods prolong the functional capacity of tissue bioimplants in the post-implantation period [8, 9]. There are also known kinds of processing the material, including that of bovine tendon, by using chemical-physical methods, including the use of saline solutions, freezing and unfreezing, soaking in a hydrogen peroxide solution with the effect of the latter on surfactants, the immersion in sugar syrup [10, 11]. It is worth paying a special attention to the use of supercritical carbon dioxide fluid (SCF-CO_2) together with surfactants (detergents), such as dodecyl sulfate and Tween-80 with ethyl alcohol, for the complete removal of organic components while maintaining the natural architectonics and strength properties of the material [12]. The method of supercritical fluid extraction is a very promising known approach to xenomaterial decellularization. The SCF-CO_2 containing a small amount of entrainer, is a suitable medium for the extraction of cell nuclei and cell membranes from synthetic material. SCF- CO_2 is capable to penetrate deeply into the material and affect cells. Under mild extraction conditions (15 MPa, 37°C), the cell nuclei can be satisfactorily removed from the tissue within 1 hour. On the contrary, the efficacy of phospholipid removal strongly depends on the rate of carbon dioxide transfer into the tissue. The mechanical strength of the tissue did not decrease even with such a long-term processing [13]. Any xenografts used as medical products must be sterilized to prevent infection transmissions. A number of studies investigating the use of SCF-CO_2 in the processing of biomaterials have indicated a great advantage of its use due to its ability to inactivate many strains of pathogenic microorganisms, viruses, and prions, potentially transmitted from the biomaterial to a human [14–16].

In connection with the above, **the aim of the study** was to develop a technique for the qualitative decellurization of the bovine tendon while maintaining and increasing its strength properties.

Material and methods

Choice of raw materials

Bovine tendon is an easily available raw material for manufacturing this kind of medical implantable products. For the manufacture of a ligament prosthesis, the flexor and extensor tendons of the bovine toe, which had passed veterinary control before the slaughter, were specially selected as having the most suitable sizes for solving the tasks set by the clinicians (Fig. 1).

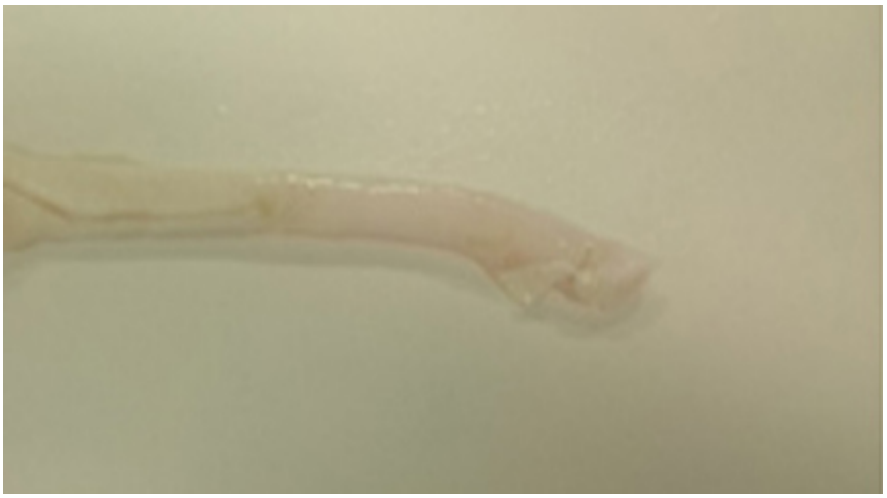

Fig. 1. Bovine toe extensor tendon before processing

Processing

The tendons were excised, the equal areas average of 0.5–1 mm in diameter were selected, mechanically cleaned from external membranes, thoroughly washed in distilled water, cut into equal lengths, then kept in a sodium chloride solution of an ascending concentration, thoroughly washed from salt, and then frozen twice with unfreezing at room

temperature, then placed in sugar syrup, blotted on a non-woven cloth, straightened along the fibers, immersed in Tween-80 emulsifier, removed and, without washing, placed in the reactor of the supercritical fluid extraction unit, processed at a given pressure and temperature; at the end of processing, the material was removed from the reactor, blotted with a dry cloth over the surface and placed in 1% hydrogen peroxide solution, washed and immersed in 0,1% solution of glutaraldehyde, after which it was again thoroughly washed with running distilled water until the foam completely ceased, froze and dried in a freeze dryer, and then was again treated with SCF-CO_2 at a given pressure and temperature.

After processing, the tendon looked as follows (Fig. 2).

Fig. 2. Bovine tendon after processing

Methods of investigation

The material was tested according to the following criteria:

- presence/absence of cellular elements;
- physical and mechanical strength properties.

Investigations for the presence of cellular elements

Fragments of bovine tendon 10x10 mm of the unprocessed material and the material processed till before the stage of stabilization in glutaraldehyde were histologically examined for the presence of cells. The histological sections obtained from the control and processed material samples were specially prepared for these examinations using fixation and staining methodology. The tissues were fixed in neutral 7% formalin solution, passed through a battery of alcohols of increasing concentration, and embedded in paraffin. Paraffin sections of 5–7 μm thick were stained with hematoxylin and eosin. Using a microscope equipped with a Sony digital camera attachment with a resolution of 12 megapixels, 5 micrographs were obtained from each histological specimen using the Image View, Image Tool v.2.00, Image Analyzer v.1.1 software. In this way the analysis for the presence of cellular elements was performed. Cross-sections were examined from each specimen. A magnification of 200x was used.

Physical and mechanical properties

An Instron 5900 testing machine was used for tensile-strength testing of the material with at least 20 repeated measurements ($n \geq 20$). The strength characteristics of the material were measured in kgf/mm^2 according to the Young's modulus. Five series of experiments were performed. The measurement results are presented as the mean and its standard error. The statistical significance of the difference was determined using the Student's t test. The following measurement results were obtained for the strength properties of the processed and unprocessed material, and are given below with the statistical significance of their differences ($p < 0.05$).

Results

Investigation for the presence of cells

Histological examination after staining with hematoxylin and eosin showed that the unprocessed material contained cells predominantly located between the bundles of collagen fibers. Cells were also found between individual fibers, but in smaller numbers. In all cases, the cells had clear-defined outlines (Fig. 3).

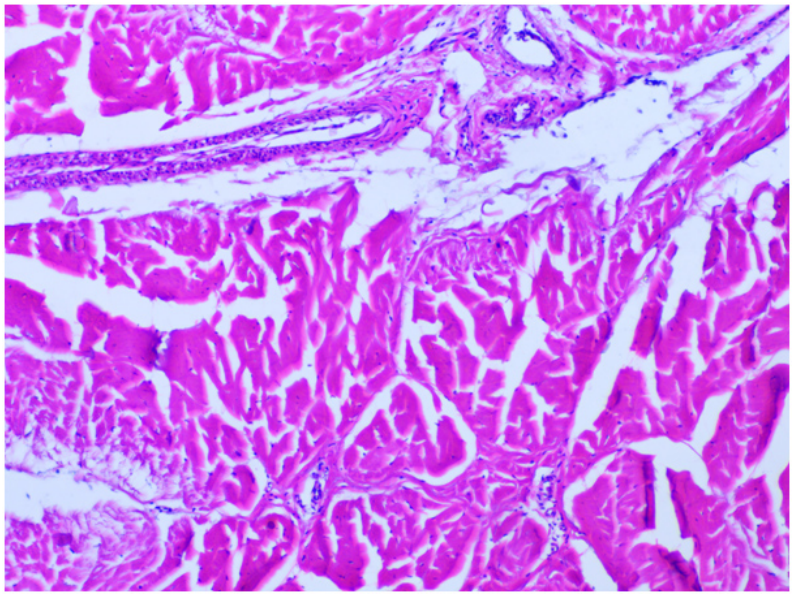

Fig. 3. Material before processing, hematoxylin and eosin staining, cross section, (magnification, x200)

The processed material after its staining with hematoxylin and eosin showed the cells were located only between bundles of collagen fibers, mainly in the area of blood vessels passing, in an amount from 0 to 2 cells in the field of view. No cells were found between individual fibers. In all cases, the cells had blurred outlines, and were partially destroyed (Fig. 4).

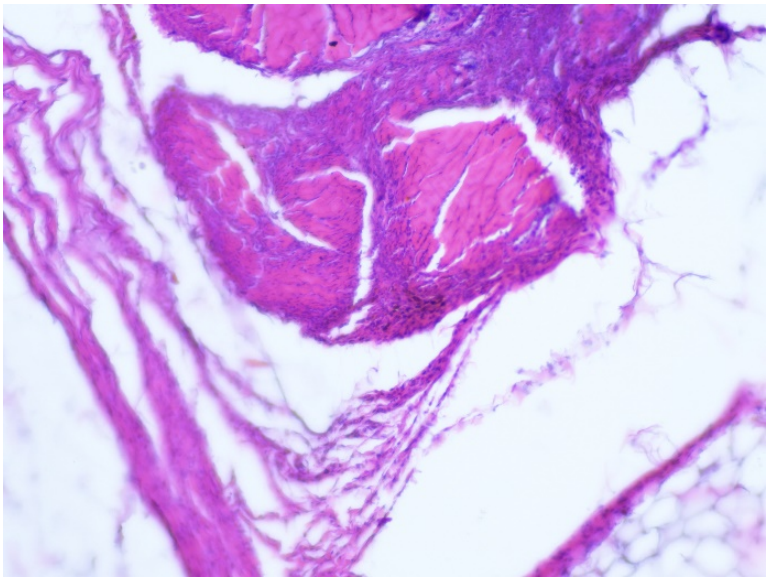

Fig. 4. Material after processing, hematoxylin and eosin staining, cross section, (magnification, x200)

Physical and mechanical properties

Physical, mechanical, and strength properties of the material before and after processing are presented in the Table as mean readings obtained for samples from every group.

Table. The results of studying the physical and mechanical properties of the material

Parameters	Ultimate load on anterior cruciate ligament [2]	Before processing	After processing
Young's modulus, kgf/mm^2	170	503±7	605±19

Discussion

Presence/absence of cells

The histological examination of the material shows that after the main stages of physical and chemical processing (till before the preservation with glutaraldehyde) aimed at decellularization of the material and elimination of biologically active substances demonstrated that there were practically no cellular components, which generally indicated the decellularization effect achieved.

Physical and mechanical properties

As can be seen from the Table, after processing, the tensile strength of the obtained material did not decrease and even became higher than that of the unprocessed material, and, in general, significantly exceeded the ultimate load for ACL. This is primarily due to the use of gentle processing modes (without aggressive chemical media), which combined moderate physical and chemical effects on the material. In addition, the increased strength of the material can also be explained by its stabilization in glutaraldehyde.

Conclusion

The applied technique of material processing gives excellent results of the bovine tendon decellularization.

The strength characteristics properties after the proposed processing of the bovine tendon have neither been lost, but have even tended to be slightly improved due to the stage of stabilization in glutaraldehyde.

The proposed processing technique is promising for the developments of a human ligament prosthesis. However, to assess the

efficacy of the material obtained, further studies of biological safety and functional properties are required in *in vitro* and *in vivo* models.

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