COMPARATIVE STUDY ON BIOMECHANICAL PROPERTIES OF THE CENTRAL PORTION OF FROZEN AND FRESH CALCANEUS TENDON

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SUMMARY

Allograft storage methods can change some mechanical characteristics of tissues. With the objective of analyzing the influence of freezing phenomenon and storage time on tendons' biomechanical properties, the authors studied 40 calcaneus tendons obtained from 20 human cadavers, with an average age of 41.95 years, ranging from 31 to 54 years old, being 17 males and three females. From each cadaver, two tendons were removed, one tested in its fresh state and the contralateral one frozen at -85º C in an electric freezer, during a period of six or 12 weeks. The bodies of evidence were submitted to traction assays in a Kratos K5002 mechanical assay machine, delivering strength-deformation graphics. Strength at maximum resistance limit, stiffness, tension at maximum resistance limit, relative deformation, and elasticity module parameters were assessed. The results were compared and statistically analyzed by "Student's t- method", with a significance level of 0.05, with no significant difference on values achieved between groups. We concluded that freezing at -85º C does not cause changes to tendons' biomechanical properties, despite of storage time.

Keywords: *Freezing; Transplantation, homologous; Achilles tendon; Biomechanics.*

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INTRODUCTION

With the growing interest on less invasive techniques development for tendinous and ligament reconstruction surgeries, the research addressed to the use of allografts has been increasing. The advantages associated to its use include a reduced operative time, lower morbidity to donator site, and the availability of a biological alternative after failures of reconstructions involving allografts. Theoretical disadvantages include high technical cost, diseases transmission and foreign body-type immune reaction potentials, and graft weakness due to preparation process[1-3]. The study of potential changes on biomechanical properties of soft tissues submitted to freezing temperatures is not new. Some authors are able to successfully reproduce allogenic tendons' transplants by means of experimental studies and surgical assessments for reconstructing ligament or tendinous injuries, whether these are chronic or acute[4-7].

An important aspect associated to the use of allografts is regarding its storage procedure. For allowing it, many tissues processing techniques have been described, including freezing, lyophilization and cryopreservation[8]. All these techniques impose significant disadvantages, both biological and biomechanical. The different methods employed for storing grafts may lead to changes in its biomechanical characteristics[9].

The Tissue Library of the Orthopaedics and Traumatology Institute, Hospital das Clinicas, Medical College, University of São Paulo, stores tissues by means of freezing procedures at -85º C and uses no additional method for sterilization, once every process that includes tissues uptake, processing and packaging is performed under aseptic conditions.

Some authors study the effects of freezing on tendons and ligaments properties by means of experimental models in animals[10]. However, there is no report in literature addressing the behavior of human tendons or ligaments submitted to freezing at – 85º C, previously to its insertion as allografts.

The objective of this study was to compare certain biomechanical properties (strength at maximum resistance limit, stiffness, tension at maximum resistance limit, relative deformation, and elasticity module) of human cadavers' Achilles tendons frozen at – 85º C to fresh Achilles tendons, and to assess the influence of storage time on those properties.

MATERIALS AND METHODS

The material of this study was constituted of 40 Achilles tendons removed from 20 human cadavers registered at the Death Examining Service of São Paulo city (SVOC-USP). Data concerning age, gender and date of death were obtained from SVOC-USP files. Ages ranged from 31 to 54 years (average: 41.95 years). From the 20 cadavers, 17 were males and three were females.

Inclusion criteria

Cadavers belonging to the age group of 17-55 years, with no previous pathologies according to SVOC-USP data, and presenting with no signs of local changes, such as wounds or scars were used. From each cadaver, tendons were removed, one serving as control (fresh) and the other submitted to freezing temperatures.

Tendons preparation

Tendons were divided into four groups of 10 units:

GC6: the control group with "fresh" tendons whose pairs were studied after 6 weeks at -85º C.

GC12: the control group with "fresh" tendons whose pairs were studied after 12 weeks at -85º C.

GE6: the study group with tendons submitted to a freezing temperature of -85º C for six weeks.

GE12: the study group with tendons submitted to a freezing temperature of -85º C for 12 weeks.

After being removed, tendons on control group (fresh) were maintained in sealed polyethylene packages in a domestic-kind refrigerator at +4 º C for a period of 24 hours until assays were performed.

Study conducted at the Orthopaedics and Traumatology Institute, HCFMUSP.

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Study group tendons were kept in three sealed polyethylene packages of 0.5 μm and frozen at a temperature of – 85° C in an electric horizontal freezer SANYO®, with CO^2 backup, temperature maintenance control by graphical monitoring and built-in alarm valve connected to an external alarm system for temperatures above – 60° C. Tendons on group GE6 were kept in freezer at – 85°C for six weeks, and tendons on group GE12, at the same temperature, for 12 weeks.

Control group preparation for assay

For control group assay, tendons were removed from the domestic-kind refrigerator at + 4° C and wrapped with gauze pieces moisturized with 0.9% physiological saline solution until bodies of evidence were prepared.

Study group preparation for assay

On the day before assay performance, frozen tendons were transferred from freezer at – 85° C to domestic-kind refrigerator at + 4° C, where they remained for 24 hours. Subsequently, they were removed and wrapped with two gauze pieces moisturized with 0.9% physiological saline solution until bodies of evidence were prepared.

Preparation of bodies of evidence

Tendons were placed extended over a wooden surface and its distal end was stabilized with the aid of a metal fixation device. Using a double-handle knife, blades 1 cm away from each other, the central strip of the tendon was removed in its longitudinal orientation.
With the aid of an ordinary knife, the tendon strip was sectioned at a cross-section plane from its longitudinal axis in its ends, so that the end length of the body of evidence was 16 cm.

Mechanical assays

All anatomical pieces were submitted to traction assays in a universal mechanical assay machine KRATOS® K5002, built with a 5000 Kg load cell. Each end of the body of evidence was fixated to a threaded sinusoidal claw with rounded edges appropriately designed for tendinous fixation, composed of two plates attached by two compression screws. After a preliminary test, five Nm value was determined for the moment of torsion to be applied on screws – required for an adequate fixation of the graft – by means of a digital METALAC® torquimeter with capacity for 100 Nm. The "distal claw" encompassed the five centimeters proximal to the body of evidence. The distance between claws before plates compression, was six cm. After compression, that distance was measured again and established as L_0. Assays were performed at a 20 mm/min speed. The machine functioning was monitored by an IBM PC® compatible computer, which captured and addressed data, building absolute and relative strength-deformation graphs.

Parameters assessed

The parameters assessed on traction assays for control and study groups were: (1) Strength at maximum resistance limit (SMRL) in Newtons; (2) Stiffness (K) in Newtons by millimeters (N/mm); (3) Tension at maximum resistance limit (TMRL) in Mega-Pascal (MPa); (4) Relative deformation (e%) as a percentage, and; (5) Elasticity module (E) in Mega-Pascal (MPa).
The determination of the cross-sectional area of the bodies of evidence was required for building tension at maximum resistance limit (TMRL) and elasticity module (E) graphs. For this reason, the bodies of evidence were positioned between a transparent support table and an acrylic block over which a steady load was applied. Then, this device was positioned at a profile projector, where we were able to obtain, by means of refracted and reflected rays projected on a screen, width and height values for the bodies of evidence. From width and height values, the cross-sectional are was calculated.
Comparative analyses of the parameters described above were performed between groups GC12 and GE12, between groups GC6 and GE6, and between groups GE12 and GE6.

Statistical treatment

We performed the descriptive statistics – average (AVG), standard deviation (SD), average standard error (ASE), maximum (MAX) and minimum (MIN) – of quantitative parameters. In the comparative analysis of parametric paired groups, the Student's paired t-test was used. For the analysis of parametric non-paired groups, we used the Student's non-paired t-test. The significance level was determined at 0.05.

RESULTS

No statistically significant difference was found in the comparative analysis of quantitative parameters between the study group with 12-week frozen tendons (GE12) and the control group (GC12) (Table 1); between the study group with six-week frozen tendons (GE6) and the control group (GC6) (Table 2); and between the study group with 12-week frozen tendons (GE12) and the group with six-week frozen tendons (GE6) (Table 3).

DISCUSSION

The outcome of a ligament or tendon reconstruction surgery is, among other factors, directly associated to the kind of graft employed. While artificial ligaments were not accepted among orthopaedic doctors, the use of autogenous tissue – certainly the most appropriate – is not exempted from complications to the donator site.
In this sense, the allograft becomes an attractive option. It can be promptly available in several kinds of tissues (Achilles tendon, patellar ligament, fascia lata, gracile and semitendinous muscles' tendons), allows for storage and, because it is prepared previously to the beginning of surgery, surgical time and the time of limb ischemia resulting from tourniquet use – when applicable – are reduced. On the other hand, the high technical cost, the risk of diseases transmission and the potential weakening of the graft as a result of the preparation process constitute a big disadvantage for the use of allografts.
According to Zimmerman et al.[8], structural disorders of collagen fibers lead to changes on biomechanical properties of grafts, and can also change its remodeling, revascularization and reintegration abilities. These disorders can also be triggered by the activity of enzymes present on tissues. At temperatures above– 40°C, some enzymes are still active, turning storage for extended periods unfeasible. On the other hand, at temperatures below – 80°C, enzymatic destruction is minimal, with at least the collagenase enzyme being inactive [11].
The intention of selecting a study involving human cadavers' tissues was to put an experimental model closer to clinical practice. Thus, we tried to reproduce the methodology employed in the use of human

Comparison between groups GE12 and GC12

Parameters	Student's t-test- Paired		Significance
SMRL	p = 0.608	t = 0.532	n.s.
K	p = 0.616	t = 0.519	n.s.
A	p = 0.397	t = 0.889	n.s.
TMRL	p = 0.225	t = 1.301	n.s.
ε%	p = 0.740	t = 0.343	n.s.
E	p = 0.167	t = 0.502	n.s.

Where: SMRL = Strength at Maximum Resistance Limit; K = Stiffness; A = Average area; TMRL = Tension at Maximum Resistance Limit; e% = Relative deformation; E = elasticity module; p = significance ratio; n.s. = non-significant. Source: IOT-HCFMUSP

***Table 1** - Comparison of quantitative parameters studied between groups ge12 and gc12. Statistical analysis by paired t-test (a = 0,05).*

Comparison between groups GE6 and GC6			
Parameters	**Student's t-test- Paired**		**Significance**
SMRL	p = 0.149	t = 1.570	n.s.
K	p = 0.059	t = 2.157	n.s.
A	p = 0.248	t = 1.235	n.s.
TMRL	p = 0.9996	t = 0.0005	n.s.
ε%	p = 0.900	t = 0.106	n.s.
E	p = 0.918	t = 0.129	n.s.

Where: SMRL = Strength at Maximum Resistance Limit; K = Stiffness; A = Average area; TMRL = Tension at Maximum Resistance Limit; ε% = Relative deformation; E = elasticity module; p = significance ratio; n.s. = non-significant. Source: IOT-HCFMUSP

Table 2 - *Comparison of quantitative parameters studied between groups ge6 and gc6. Statistical analysis by Student's paired t-test (α = 0,05).*

Comparison between groups GE12 and GE6			
Parameters	**Student's t-test- Non-Paired**		**Significance**
SMRL	p = 0.379	t = 0.901	n.s.
K	p = 0.205	t = 1.169	n.s.
A	p = 0.243	t = 1.207	n.s.
TMRL	p = 0.569	t = 0.581	n.s.
ε%	p = 0.932	t = 0.087	n.s.
E	p = 0.387	t = 0.887	n.s.

Where: SMRL = Strength at Maximum Resistance Limit; K = Stiffness; A = Average area; TMRL = Tension at Maximum Resistance Limit; ε% = Relative deformation; E = elasticity module; p = significance ratio; n.s. = non-significant. Source: IOT-HCFMUSP

Table 3 – Comparison of quantitative parameters studied between groups ge12 and ge6. Statistical analysis by Student's non-paired t-test (α = 0,05).

allografts, including the kind of tissue, manipulation details and the dimension of the bodies of evidence. We used the Achilles tendon for this study due to the large number of authors who reported its use in different kinds of tendon and ligament reconstructions [4-7].

Since the key role of tendons and ligaments is to transfer tensile load, experimental studies of the biomechanical properties of these tissues are, in general, performed by means of traction assays. The objective of those tests is to capture strength-deformation graphs, from which mechanical properties are determined. The traction assay consists of submitting a material to a strength tending to stretch or elongate it. The assay is usually performed in a body of evidence with standard formats and dimensions, for being able to compare the results achieved, or, if required, to reproduce them. Yet, traction tests may be subjected to bias caused by several factors that lead to experimental artifacts with resultant results reliability loss. When reviewing their complications, Nikolaou et al.[1] note that, both the standardization of graft dimensions (especially the cross-sectional area measurement) and its stable fixation onto the claws, are important factors for an overall successful surgery.

Our results are consistent with those described by other authors. Barad et al.[12] didn't find significant differences between the mechanical properties of ACL in Rhesus monkeys' knees, either those frozen at – 80° C or fresh ones, after a period of three to five weeks. Bechtold et al.[13] compared the effects of freezing processes at – 70° C and lyophilization over the mechanical characteristics of human patellar tendons, suggesting that the resistance of frozen tendons was superior to lyophilized tendons'. Similarly, the storage of tissues at –20° C has shown to be appropriate, according to Woo et al.[10], for keeping the mechanical characteristics of rabbits' ligaments. Nevertheless, the same author recommends that tissues submitted to freezing temperatures above – 40° C, should not be stored for an extended period of time, due to a potential enzymatic action and cell disorder. We understand that the difficulty in comparing the results achieved to those described by literature is due to the heterogeneous nature of assays. In the only study addressing human cadavers' patellar ligaments (14), the authors use freezing temperatures of – 20° C, with no standardization of the bodies of evidence. In the study involving freezing at – 80° C, musculotendinous units of animal models with characteristics that are distinct from human Achilles tendon[12].

The scientific relevance of this study is based on the creation of the Hospital das Clínicas' Tissues Library, University of São Paulo. Proving the results described in literature about biomechanical changes on tendons or ligaments frozen at –85o C was required, since most references were based on results achieved from few studies with animal models[1,12].

We regard the freezing method at – 85° C as appropriate for storing soft tissues designed to transplantations, since its mechanical properties were not changed despite of the storage time. However, further studies are warranted, aiming to assess the influence of this method on the process of biological incorporation of grafts regarding cell repopulation, revascularization and collagen remodeling phenomena, as well as on the mechanism of incorporation of the graft into bone tunnel, potentially damaged by the stretching resulting from immune response.

CONCLUSIONS

We concluded that the process of freezing at – 85° C does not cause changes to biomechanical properties of tendons, despite of the time of freezing.

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