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Selection of an optimal cryoprotectant for long-term storage of human tendon allografts

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Abstract

Introduction. *Tendon grafts are widely demanded in reconstructive plastic surgery. Allogeneic tendons potentially have a number of advantages. However, the method of long-term storage of allogeneic tendons has not been optimized to date.*

Aim. *Selection of an optimal cryoprotectant for the storage of human tendon allografts at ultra-low temperatures, which allows preserving the native tissue structure.*

Material and methods*. We studied M. tibialis anterior tendon grafts taken from tissue donors. Endocellular/penetrating cryoprotectants (dimethyl sulfoxide, polyethylene glycol-400, glycerol) and exocellular/non-penetrating cryoprotectants (glucose solution, albumin solution) were used in the cryopreservation process. Tendon mechanical*

properties were evaluated using rupture and stretching-shear test, the general morphology of tendons, topography, density and integrity of collagen fibers, preservation of cellular elements were microscopically evaluated.

Results. *Histological analysis showed that the safety of collagen and elastin fibers differed depending on the cryoprotectant used. At the same time, micro-fractures of collagen fibers were microscopically detected in all the experiments. In the presence of dimethyl sulfoxide, polyethylene glycol-400 and their combination the structure of collagen fibers and cells did not undergo visible changes compared to the control, whereas in all the experiments with non-penetrating cryoprotectants the topography and orientation of the fibers were clearly disturbed, deformation of many cells in the tendons was also observed.*

Conclusions. *Cryoprotectants based on dimethyl sulfoxide, polyethylene glycol and their combinations allowed us to preserve the structural integrity of allogeneic tendons. Non-penetrating cryoprotectants did not effectively preserve the integrity of collagen fibers and cells in the tendons and cannot be recommended for cryopreservation.*

Keywords: cryopreservant, tendon, freezing, allograft

Conflict of interests Authors declare no conflict of interest

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CMI cell membrane integrity
DMSO - dimethyl sulfoxide
MMSC - multipotent mesenchymal stromal cells
PEG - polyethylene glycol

Introduction

The problem of preservation of allogeneic human tissues is of increasing interest, both from a practical and scientific point of view. The main task of preservation is the long-term storage of grafts, which allows preserving the native tissue structure. [1]. When preserving allogeneic tissues containing no living cells (bone, dura mater, pericardium), the objective of all methods is to preserve the overall topography of the tissue and its mechanical properties [2]. Preservation of tissues containing viable cells (tendons, cartilage, skin) requires a much more delicate approach and is technologically more complex [3]. This explains the fact that today there are no truly effective methods of long-term storage of allogeneic tendons. Tendon grafts are widely in demand in reconstructive plastic surgery [4], while the use of allogeneic tendons has a number of advantages compared to autologous material: no injury to the donor site, shorter duration of surgical intervention, less postoperative pain, the possibility of in-advance production of the required size grafts [5, 6]. For preserving the functional and biomechanical properties, viable cells must be constantly present in the tendons, synthesizing the tendon extracellular matrix, primarily collagen. In this regard, the methods of preserving cell-free collagen matrices, which are widely available in clinical practice, are unacceptable when working with tendon grafts [7]. To date, the only adequate approach to long-term storage of tendons is their cryopreservation. When working with tendon grafts, the following factors must be taken into account: sterility, autolysis prevention, and preservation of plastic, structural, and functional properties [8].

When examining tendon grafts, it is necessary to conduct a comprehensive study, which includes a morphological analysis of cells and fibers in tendons, as well as their biomechanical properties [9].

The aim of this work is to choose an optimal cryoprotectant for storing human tendon allografts at ultra-low temperatures, which allows preserving the native tissue structure.

Material and methods

The pilot study included 80 grafts of m. tibialis anterior tendon taken from tissue donors.

The graft explantation operation was performed in the Operating Room in compliance with the rules of asepsis and antisepsis. The surgical access was performed by making linear incisions of 2 cm on the anterior surface of the lower leg and foot in the projection of the joint space of the ankle joint and tendon entheses. The tendons were mobilized, their length being measured, and cut off from the attachment sites. After that, all grafts were mechanically cleaned from adjacent tissues, placed in sterile bags with an antibiotic solution (gentamicin 4%, 2 ml, or vancomycin 1000 mg) and were quarantined at a temperature of +4°C until the results of the analysis for the presence of transmissible infections had been obtained. Then the tendons were washed in physiological saline solution of sodium chloride 0.9% for 5-10 minutes in a laminar cabinet in compliance with the rules of asepsis and antisepsis. After that, the tendons were placed in cryopackages, 50 ml of a cryopreservation solution was added, the packages were sealed, labeled, and placed in a freezer at a temperature of -80^{o}C.

Depending on the type of cryoprotectant, four groups for comparison were formed: group 1 included untreated, native tissue grafts (control), which had not been exposed to cryoprotectants or freezing. Tendon grafts treated with penetrating (group 2) or non-penetrating cryoprotectants (group 3), as well as with their combination (group 4),

which were frozen and stored (Table), comprised other three study groups.

Table. Types of cryoprotectants used for tendon cryopreservation

<table>
<tr><th>1st group: without cryoprotectants (control)
8 pcs.</th><th>2nd group: penetrating cryoprotectants
24 pcs.</th><th>3rd group: non-penetrating cryoprotectants
32 pcs.</th><th>4th group: a combination of cryoprotectants
16 pcs.</th></tr>
<tr><td rowspan="4">8 tendons of m. tibialis anterior dextra et sinistra, neither cryopreserved nor frozen</td><td rowspan="2">2.1)10% DMSO solution, 8 pcs.</td><td>3.1) 10% glucose solution, 8 pcs.</td><td rowspan="2">10% PEG + 5% DMSO, 8 pcs.</td></tr>
<tr><td>3.2) 15% albumin solution, 8 pcs.</td></tr>
<tr><td>2.2) 15% PEG-400 solution, 8 pcs.</td><td>3.3) 20% albumin solution, 8 pcs.</td><td rowspan="2">Glycerol 57, 1% + 15% PEG-400, 8 pcs.</td></tr>
<tr><td>2.3) 10% PEG-400 solution, 8 pcs.</td><td>3.4) 5% glucose solution, 8 pcs.</td></tr>
</table>

Notes: DMSO − dimethyl sulfoxide; PEG − polyethylene glycol

All tendons of groups 2–4 were frozen and stored at -80°C for 20 days. After defrosting, the tendons were washed with isotonic physiological 0.9% sodium chloride solution, their mechanical properties, toxicity, and morphology on histological sections were assessed.

To study the mechanical characteristics, all tendon samples were tested using a Metrotex tensile testing machine using a sensor up to 70 kg and a dynamic mechanical analyzer. The load-dependence on tension parameters, as well as the conditional deformation modulus were evaluated. Each sample was tested for rupture and tension-shear at least 3 times.

The toxicity study of tendon grafts was performed in a multipotent mesenchymal stromal cell (MMSC) culture at the 3rd–9th passage isolated from the bone marrow of tissue donors. Fragments of tendon grafts 1.0–1.5 cm long were placed in experimental wells of a 6-well plate and a suspension containing 50,000 MMSCs was added. In parallel,

50,000 MMSCs were injected into wells without tendon grafts (control). Cells were cultured in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C and 5% CO_2 concentration for 3 days. For microscopic analysis, the cells were stained with a vital fluorochrome dye based on tripaflavin and rhodamine C [10]. The total count of cells ($10^3/cm^2$), their morphology were assessed, and the cell membrane integrity (CMI) was scored (in points).

For tissue microscopic study, 1x1 cm samples were taken from all grafts, fixed with 10% formalin solution, and histological specimens were prepared according to the standard method. Paraffin sections 5 μm thick were made using a rotary microtome, then stained with hematoxylin and eosin, picrofuchsin according to Van Gieson technique; 88 micropreparations were investigated, which were studied by light optics using an Olympus CX21 light microscope. In addition, autofluorescence level of their collagen fibers was assessed in histological preparations ($\lambda_{excitation}$: 510–560 nm, $\lambda_{emission}$: from 575 nm, exposure time: 1 s) according to the method developed by M.S. Makarov, Cand. Biol. Sci. [11].

Results

The evaluation of the mechanical properties of the grafts showed that in all groups, the tendons retained their integrity under the impact of the tensile force and the forces aimed at compression-shear. It must be admitted that in our work, the ultimate rupture force made 50–70 kg, which may be insufficient to assess the strength of the tendons. However, in all experimental groups, there was no complete rupture of the tendons after modeling the three-time stretching of the samples with the maximum force of the tensile machine, which indicated sufficient strength characteristics and the possibility of multiple overstretching without compromising the integrity of the collagen fibers. It follows that

the stability of mechanical properties is maintained during repeated cyclic force impacts on tendon graft samples.

The investigation in vitro showed that all studied types of grafts imposed no toxic effect on cells. After 3 days of cultivation, a subconfluent monolayer with a cell density of 17-18 thousand/cm^2 formed in the control and all experimental wells. MMSCs had a typical spindle shape, the CMI level in all the experimental ones was scored 34–36 and did not differ statistically significantly from that in the control. Thus, allogeneic tendon grafts after cryopreservation using different types of cryoprotectants were non-toxic.

Histological analysis showed that the safety of collagen and elastin fibers differed depending on the cryoprotectant used. In experiments where penetrating cryoprotectants were used: DMSO 10%, PEG-400 10%, and 15%, the overall architecture of the fibers (their orientation, interaction with each other and with cells) did not undergo visible changes compared to the control (Fig. 1A, 1B). When using non-penetrating cryoprotectants (group 3), the topography and orientation of the fibers were disturbed (Fig. 1C). The morphological structure of the tendon after cryopreservation in a combination of cryoprotectants DMSO and PEG-400 (group 4) was little different from that of the native tendon, the fibers were well-organized, unidirectional, slightly edematous with a minimum number of tissue rupture areas (Fig. 1D). On the contrary, the tendons treated with the combination of glycerol with PEG-400 differed histologically from the control samples: the fibers were edematous and the layers of loose fibrous connective tissue between the bundles (peritenonium) were expanded, areas of tissue ruptures were visualized over the entire preparation.

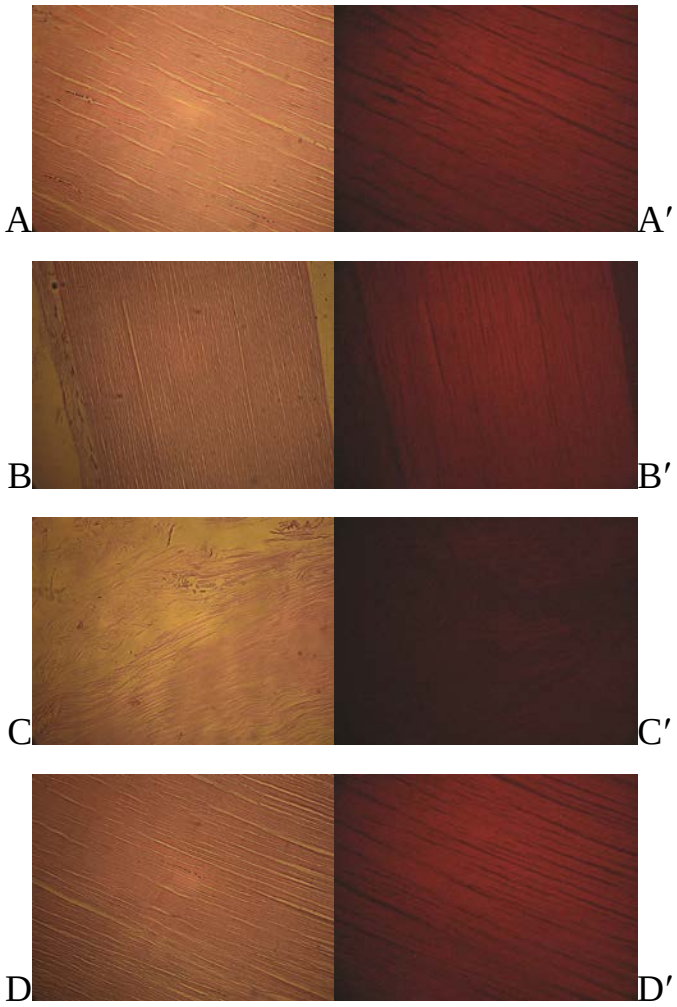

Fig. 1. Evaluation of collagen fiber integrity after cryopreservation of allogeneic tendons by various methods. On the left, hematoxylin-eosin staining; on the right, collagen autofluorescence. Magnification x200. A, A′ – control (native tendons); B, B′ – 2nd group (penetrating cryoprotectants) using 10% dimethyl sulfoxide solution as an example; C, C' – 3rd group (non-penetrating cryoprotectants) using 20% albumin solution as an example; D, D´ – 4th group (combination of cryoprotectants) using a solution of dimethyl sulfoxide and polyethylene glycol-400 as an example

Microscopic ruptures of collagen fibers were observed in all groups, except for the control group. In experiments where DMSO and PEG-400 were used, the mean rupture width was no more than 25 μm (Fig. 2A), while in the experiments with glucose and albumin solutions,

the fiber rupture width exceeded 40 μm (Fig. 2B). When using a combination of cryoprotectants, the presence of fiber breaks in the tendon samples was also noted. Meanwhile, the combination of PEG with DMSO turned out to be preferable (Fig. 2C). The fiber breaks were solitary, and their width did not exceed 25 μm. When using 57.1% glycerol in combination with PEG-400, multiple ruptures ranging in size from 40 to 70 μm were noted over the entire width of the collagen fibers; the morphological structure of the dense fibrous connective tissue was disturbed, and swelling of the collagen fibers was observed.

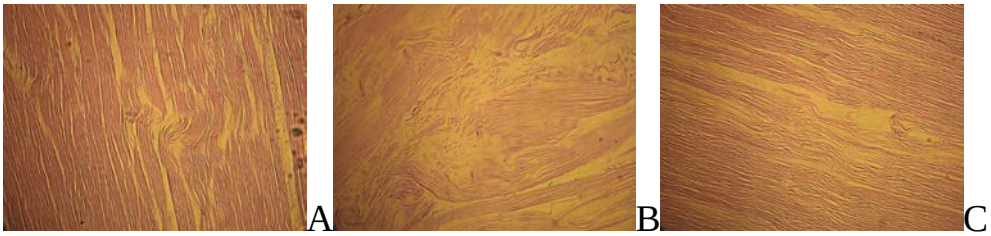

Fig. 2. Detection of ruptures of collagen fibers in allogeneic tendons after cryopreservation. On the left, hematoxylin-eosin staining; on the right, collagen autofluorescence. Magnification x200. A – 2nd group (penetrating cryoprotectants) using a 10% solution of dimethyl sulfoxide as an example; B – 3rd group (non-penetrating cryoprotectants) using a 20% albumin solution as an example. C – 4th group (combination of cryoprotectants) using a solution of dimethyl sulfoxide and polyethylene glycol-400 as an example

The collagen fluorescence intensity along the entire length of the fibers corresponded to the norm in the control and in experiments with either 10% DMSO or PEG-400 (group 2), making a mean of 39±2 foot-candles (Fig. 1B´). In the group of using the combination of PEG-400 with DMSO (group 4), most of the fibers had normal autoluminescence intensity and made a mean of 35±2 foot-candles (Fig. 1D´). On the contrary, in the 3rd group, a significant part of the fibers had a very low autoluminescence intensity (from 7 to 15 foot-candles), the mean value of

autofluorescence was 19±1 foot-candles (Fig. 1C´). At the same time, in the grafts of the 3rd group, a pronounced decondensation of collagen fibers with disintegration into separate fibrils.

In all groups, including the control one, there were areas with pronounced swelling of the fibers; the most extensive such areas were noted when using albumin 15 and 20%. The swelling of the fibers may be related to the quarantine period when the tendons were in an antibiotic solution for 24 hours. The swelling of the fibers was not accompanied by an impairment of the collagen structure. In all samples, there were no fibers with an autoluminescence intensity above 60 foot-candles (picrinophilic fibers with pronounced signs of chemical destruction of collagen). The morphology of tendinocytes in the control, the 2nd, and 4th groups was normal, while in the 3rd group, many cells were shrunken and their nuclei were deformed (Fig. 3 A–D).

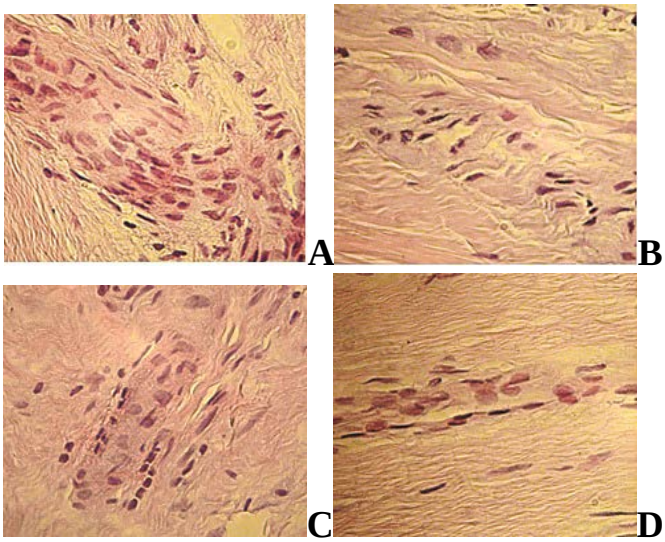

Fig. 3. Evaluation of the cellular component of allogeneic tendons after cryopreservation by various methods. Hematoxylin-eosin staining. Magnification x500. A − control (native tendon); B − 2nd group (penetrating cryoprotectants) using a 10% dimethyl sulfoxide solution as an example; C − 3rd group (non-penetrating cryoprotectants) using a 15% albumin solution as an example; D − 4th group (combination of cryoprotectants) using a solution of dimethyl sulfoxide and polyethylene glycol-400 as an example

Thus, the success of the clinical use of allografts for plastic surgery of the human:ligamentous structures largely depends on the choice of the tendon preservation method. Collagen has a high ability for self-organization and autohesion [12], while at the same time, a properly selected protectant is needed for a long-term storage of tendons, which will not damage tissue structures [13]. In this regard, the most promising method for preserving the native structure of tendons, from our point of view, is their preservation at ultralow temperatures using cryoprotectants. However, after analyzing the world literature data, we can argue that there are no generally accepted methods for tendon preservation, which means that this topic is still relevant and requires further study [14]. According to the results of the conducted experiment, we can say that the most preferable is the use of penetrating cryoprjtectants or their combination. The use of non-penetrating cryoprotectants, such as albumin or glucose, does not allow preserving both the integrity of collagen fibers and the preservation of cellular elements of dense fibrous connective tissue.

Conclusions

1. Non-penetrating cryoprotectants do not allow maintaining the integrity of collagen fibers and cells in tendons and cannot be recommended for cryopreservation.

2. Penetrating cryoprotectants: a 10% solution of dimethyl sulfoxide and polyethylene glycol-400, as well as a combination of 10% polyethylene glycol + 5% dimethyl sulfoxide most carefully preserve the structure of collagen fibers and cellular components, while not affecting the mechanical characteristics of the tissue, and therefore can be recommended as optimal protectant solutions for a long-term storage of native tendons.

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