Materials Science and Engineering C 75 (2017) 43–49

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Terminal sterilization of equine-derived decellularized tendons for clinical use

Alessandro F. Pellegata [a,1], Marta Bottagisio [b,1], Federica Boschetti [a], Marco Ferroni [a], Monica Bortolin [c], Lorenzo Drago [c,d], Arianna B. Lovati [b,*]

[a] Department of Chemistry, Materials and Chemical Engineering Giulio Natta, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy
[b] Cell and Tissue Engineering Laboratory, IRCCS Galeazzi Orthopaedic Institute, Via Riccardo Galeazzi 4, 20161 Milan, Italy
[c] Laboratory of Clinical Chemistry and Microbiology, IRCCS Galeazzi Orthopaedic Institute, Via Riccardo Galeazzi 4, 20161 Milan, Italy
[d] Department of Biomedical Science for Health, University of Milan, via Luigi Mangiagalli 31, 20133 Milan, Italy

ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 2 February 2017
Accepted 5 February 2017
Available online 10 February 2017

Keywords:
Sterilization
Decellularized tendons
Peracetic acid
Irradiations
Collagen
Biomechanical properties

ABSTRACT

In the last few years, the demand for tissue substitutes has increased and decellularized matrices has been widely proposed in the medical field to restore severe damages thanks to high biocompatibility and biomechanical properties similar to the native tissues. However, biological grafts represent a potential source of contamination and disease transmission; thus, there is the need to achieve acceptable levels of sterility. Several sterilization methods have been investigated with no consensus on the outcomes in terms of minimizing structural damages and preserving functional features of the decellularized matrix for transplantation in humans.

With the aim of making decellularized tendons safe for clinical use, we evaluated the cytocompatibility, and biochemical, structural and biomechanical variations of decellularized equine tendons sterilized with peracetic acid or β-irradiation and differently wet- or dry- stored at 4 °C or −80 °C, respectively.

Considering that both sterilization and long-term storage are crucial steps that could not be avoided, our results pointed at ionizing β-rays as terminal sterilization method for decellularized grafts followed by frozen dry storage. Indeed, this approach can maintain the integrity of collagen-based structures and can avoid biomechanical changes, thus making xenogeneic decellularized tendons a promising candidate for clinical use.

1. Introduction

Severe tendon ruptures require the replacement of the lacking tissue using autografts [1], allografts [2] or xenografts. In the last few years, the demand for tendon substitutes has increased with over 15 million injuries in the US related to trauma, tumors or atrophy [3]. Considering these aspects, decellularization techniques of animal-derived tendon graft have been investigated to obtain substitutes featuring high biocompatibility, extracellular matrix support and biomechanical properties similar to the native tissue [4]. The decellularization process allows the removal of donor cells and antigen debris avoiding any adverse immune response of the graft recipient [5,6]. However, xenografts represent a potential source of infections. In particular, non-sterile conditions during harvest and manipulation could influence the levels of bioburden in these tissues, while for synthetic material devices microbial contamination can be kept to low levels [7]. With the aim of making biological materials safe for transplantation, several sterilization methods are commonly used in practice, such as ionizing irradiations (γ and β-irradiation), ethylene oxide (EtO) or processes by means of other organic compound (peracetic acid, PAA). Currently, γ-irradiation at 25 kGy is the most popular sterilization technique for medical devices and it can provide a Sterility Assurance Level (SAL) of 10^{-6} [7]. However, it has been demonstrated that γ-irradiation could decrease the mechanical strength of collagen-containing tissues, it could damage the cross-linking of the collagen, and it could increase the resistance toward enzymatic degradation [8–10]. The EtO treatment achieved popularity as a sterilization method for biological specimens because of its good monitoring control and because this process can be performed at lower temperatures compared to irradiations. However, EtO is a very hazardous alkylating agent associated with carcinogenic, mutagenic, and irritating residuals after evaporation that could cause severe chronic inflammations [8,11], and also it could affect the tensile strength of collagen structure [12]. On these grounds, the aim of this study is to

Abbreviations: Peracetic acid, PAA; ethylene oxide, EtO; superficial digital flexor tendons, SDFT; phosphate buffer saline, PBS; room temperature, RT; tri-n-butyl phosphate, TBP; glycosaminoglycans, sGAG; Tryptic Soy Agar plates, TSA; Brain Heart Infusion broth, BHI; Dithiothreitol, DTT; 1,9-dimethylmethylene blue, DMMB; decellularized matrix, DM; Haematoxylin and Eosin, H&E; elastic modulus, EM; peak test, E_p; relaxation test, E_r.

* Corresponding author.
E-mail address: arianna.lovati@grupposandonato.it (A.B. Lovati).

[1] Equally contributed.

http://dx.doi.org/10.1016/j.msec.2017.02.009
0928-4931/

evaluate β-irradiations and PAA as decontamination methods of decellularized equine tendons that were previously investigated as scaffolds for tissue engineering [13] and to treat them with a sterilization method suitable for a potential clinical use. In fact, in our previous study, we demonstrated that equine tendons decellularized with 1% Tri-n-butyl-phosphate and 3% PAA preserved their biomechanical properties and they were cytocompatible, but no further sterilization techniques were investigated [13]. However, a terminal method of sterilization suitable for decellularized tissues should be provided for clinical use. Thus, β-irradiation was investigated because of its lower penetration and dosage compared to γ-irradiation, hypothesizing a reduced effect on biomechanical properties and a minimized damage of the final device. Moreover, since in our previous study [13], PAA was used as the final step, we wanted to verify if this oxidizing agent can provide decellularized devices free of microorganisms, thus avoiding the need of any irradiation process. Indeed, it is well recognized that PAA can provide a complete sterilization of bone and cardiac valve allografts in terms of viral, bacterial and fungal pathogens [14,15].

Beside sterilization, shelf storage should be also investigated when aiming at a potential clinical use of decellularized scaffolds. Different studies proposed various solutions involving cold temperatures [16,17]. To this aim, we stored the decellularized scaffolds at 4 °C and at −80 °C for two months after the sterilization process.

Overall, the purpose of this *in vitro* study was to evaluate the cytocompatibility, and biochemical, structural and biomechanical variations of decellularized equine tendons sterilized with PAA or β-irradiation and differently wet- or dry-stored at 4 °C or −80 °C, respectively.

2. Materials and methods

2.1. Equine tendon retrieval

Equine superficial digital flexor tendons (SDFT) were collected from four different adult horses (n = 4; mean age 10 ± 6 years) at the slaughterhouse. Four longitudinal slices - sized 100 mm length × 15 mm width × 3 mm thickness – were obtained from each tendon, and stored at −80 °C until use.

2.2. Protocol of decellularization

After thawing in phosphate buffer saline (PBS) at room temperature (RT) for 30 min, the slices were decellularized as previously described in another study [13]. Briefly, the samples were immersed in 1% tri-n-butyl phosphate (TBP) buffered in 1 M Tris-HCl pH 7.8 for 24 h at RT under agitation, rinsed twice in ddH_2O at RT for 15 min and stored in PBS at 4 °C for 24 h to remove residual detergents. After the immersion in 0.0025% DNAse-I in PBS at RT under agitation for 4 h, the specimens were incubated in 3% aqueous solution of peracetic acid (PAA; stock solution 32%) under agitation at RT for 4 h. Finally, slices were rinsed twice in ddH_2O for 15 min, twice in PBS for 15 min, and then differently treated for decontamination.

2.3. Protocols of decontamination

To evaluate both the efficacy of the decontamination and the preservation of the decellularized matrix integrity after the sterilization and long term storage, one slice per donor (n = 4) was differently treated as follows: (1) refrigerated storage at 4 °C in PBS (RS); (2) frozen dry with excess water removed at −80 °C (FD); (3) β-irradiation followed by refrigerated storage at 4 °C in PBS (βIR + RS), and (4) β-irradiation followed by frozen dry with excess water removed at −80 °C (βIR + FD). In particular, for β-irradiation, the decellularized matrices were submerged in PBS in 50 ml tubes and irradiated with intensities of 15 kGy using a 10 MeV source (Bioster S.p.A., Seriate, BG, Italy). All the samples were stored for two months, and then analyzed in duplicate for bacterial detection, glycosaminoglycans (sGAG) and collagen quantification, histological morphology and proteoglycan integrity, and biomechanical properties. The sample partition and the experimental design are reported in Fig. 1.

2.4. Cytotoxicity and cytocompatibility tests

Immediately after the decellularization and decontamination, and prior to storage, decellularized matrices (DM) and matrices decontaminated by means of β-irradiation (DM + βIR) were tested *in vitro* for cytotoxicity under indirect culture with NIH-3 T3 murine fibroblasts and for biocompatibility. Specifically, NIH-3 T3 cells were seeded $3 \times 10^3/cm^2$ in 6-well plates in complete medium (CM) consisting of Dulbecco's modified Eagle's medium high glucose (Gibco, Life Technologies, Monza, Italy), 10% fetal bovine serum (FBS, Hyclone, Life Technologies, Monza, Italy), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 1% sodium pyruvate and 1% HEPES (all from Gibco, Life Technologies, Monza, Italy). NIH-3 T3 fibroblast monolayer was indirectly cultured in presence of DM or DM + βIR samples (5 × 5 × 3 mm pieces) placed in 0.4 μm pored Transwell® inserts (Corning, Corning, NY, USA) and incubated at 37 °C in 5% CO_2. After 72 h, the cell viability was assessed by a colorimetric assay - 3(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium-bromide (MTT, Sigma-Aldrich, Milan, Italy). Briefly, 2 ml of MTT diluted in DMEM without phenol red (Gibco, Life Technologies, Monza, Italy) (0.5 mg MTT/ml, dilution 1:10) were added to each well containing the cell monolayer and incubated at 37 °C for 4 h. After the incubation period, the formazan crystals produced by living cells were solubilized with 1 ml of pure isopropanol for 30 min at RT under agitation. The absorbance was read at 570 nm by means of a microplate reader (Victor ×3, Perkin Elmer, Waltham, MA, USA). Three biological replicates were evaluated and compared to NIH-3 T3 fibroblasts cultured in fresh CM (negative control, NC) or in

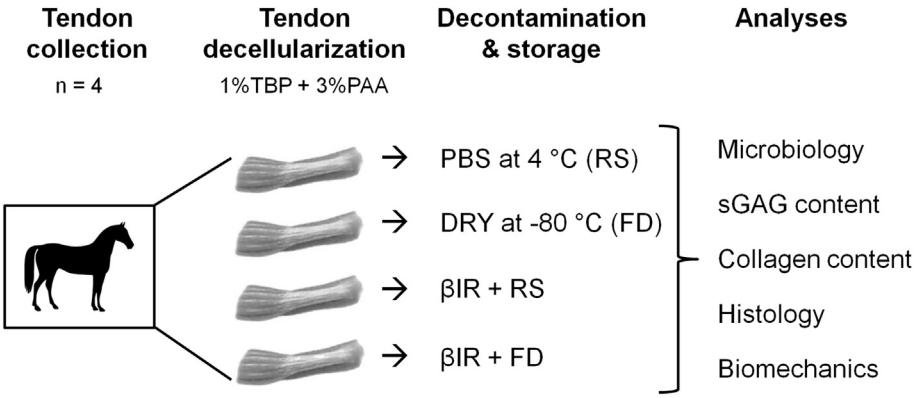

Fig. 1. Experimental study design.