Archives of Oral Biology 88 (2018) 67–76

Contents lists available at ScienceDirect

Archives of Oral Biology

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

# Assessment of static and perfusion methods for decellularization of PCL membrane-supported periodontal ligament cell sheet constructs

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ARTICLE INFO

*Keywords:*
Tissue engineering
Periodontal ligament
Cell sheet
Decellularization
Melt electrospinning
Polycaprolactone

ABSTRACT

*Objectives:* Decellularization aims to harness the regenerative properties of native extracellular matrix. The objective of this study was to evaluate different methods of decellularization of periodontal ligament cell sheets whilst maintaining their structural and biological integrity.

*Design:* Human periodontal ligament cell sheets were placed onto melt electrospun polycaprolactone (PCL) membranes that reinforced the cell sheets during the various decellularization protocols. These cell sheet constructs (CSCs) were decellularized under static/perfusion conditions using a) 20 mM ammonium hydroxide (NH4OH)/Triton X-100, 0.5% v/v; and b) sodium dodecyl sulfate (SDS, 0.2% v/v), both +/− DNase besides Freeze–thaw (F/T) cycling method. CSCs were assessed using a collagen quantification assay, immunostaining and scanning electron microscopy. Residual fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were assessed with Bio-plex assays.

*Results:* DNA removal without DNase was higher under static conditions. However, after DNase treatment, there were no differences between the different decellularization methods with virtually 100% DNA removal. DNA elimination in F/T was less efficient even after DNase treatment. Collagen content was preserved with all techniques, except with SDS treatment. Structural integrity was preserved after NH4OH/Triton X-100 and F/T treatment, while SDS altered the extracellular matrix structure. Growth factor amounts were reduced after decellularization with all methods, with the greatest reduction (to virtually undetectable amounts) following SDS treatment, while NH4OH/Triton X-100 and DNase treatment resulted in approximately 10% retention.

*Conclusions:* This study showed that treatment with NH4OH/Triton X-100 and DNase solution was the most efficient method for DNA removal and the preservation of extracellular matrix integrity and growth factors retention.

## 1. Introduction

Tissue engineered cell sheet technology has gained attention as a promising technique in the field of regenerative medicine (Dan et al., 2014; Flores, Hasegawa et al., 2008; Flores, Yashiro et al., 2008; Ishikawa et al., 2009; Vaquette et al., 2012; Zhao et al., 2013; Zhou et al., 2007). Indeed, several pre-clinical studies have shown that this approach is very promising for promoting periodontal regeneration, through the delivery of periodontal ligament cell sheets at the root surface (Flores, Yashiro et al., 2008; Ishikawa et al., 2009). However, there are few underlying limitations hindering this technology from being applicable in clinical practice. A significant issue is the reliance on an appropriate cell source in terms of functionality and adequate cell numbers, with autogenous sources hampered by patient morbidity and heterogeneity in regenerative capacity, while allogeneic sources are associated with safety concerns. There are also the issues of dedicated cell culture facilities, technical expertise, transport and associated costs.

Decellularization is a strategy that could be utilized to overcome the potential limitations to applying cell sheet technology to the clinical setting by removing the necessity of implanting constructs containing

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https://doi.org/10.1016/j.archoralbio.2018.01.014
Received 24 October 2017; Received in revised form 27 December 2017; Accepted 18 January 2018

viable cells. The effectiveness of decellularized tissues and organs has been widely reported in regenerative medicine applications, showing that biological and mechanical properties are retained following the decellularization process without eliciting an adverse immunogenic response when implanted *in vivo* (Burk et al., 2014; Nonaka et al., 2014; Syed, Walters, Day, Kim, & Knowles, 2014; Weber et al., 2013; Xiong et al., 2013; Zhang, Zhang, & Shi, 2013). Aside from the use of native decellularized tissues and organs, tissue engineered decellularized constructs prepared *in vitro* have been shown to retain their structural integrity and maintain their molecular functionality (Elder, Eleswarapu, & Athanasiou, 2009), as well as enhance tissue regeneration when used *in vivo* (Sadr et al., 2012). Decellularization has the potential to have significant implications for the commercialization of tissue engineered constructs by facilitating the development of 'off-the-shelf' products. Indeed, decellularized allografts and xenografts such as Alloderm® and Mucograft® are already commercially available for clinical use in a variety of fields, including periodontics (Bloch et al., 2011; Dijkman, Driessen-Mol, Frese, Hoerstrup, & Baaijens, 2012; Fang et al., 2007; Flynn, Semple, & Woodhouse, 2006; Hoshiba, Lu, Kawazoe, & Chen, 2010; Shimizu et al., 2007; White, Agnihotri, Titus, & Torchiana, 2005; Yazdani et al., 2009).

The use of 3D biomimetic bioprinted constructs as well as polymer supported fibrous cell sheetconstructs may be particularly suited to the regeneration of soft-hard tissue interfaces, such as the fibrocartilage phase (Gurkan et al., 2014) and the complex (bone-ligament-cementum) structure of periodontal attachment (Noppe, Roberts, Yap, Gomez, & Neufeld, 2015; Ahn et al., 2015; Akizuki et al., 2005; Dan et al., 2014; Ma et al., 2012; Vaquette et al., 2012; Vaquette, Ivanovski, Hamlet, & Hutmacher, 2013). However, the utilization of decellularized periodontal ligament cell sheets has only recently been reported in the literature (Farag, Vaquette, Hutmacher, Bartold, & Ivanovski, 2017; Farag et al., 2014) and presents some unique challenges, with the inherent fragility of the sheets presenting issues with handling and delivery. These challenges are accentuated in the context of decellularization, which is a technique which requires considerable handling and manipulation. The use of a thin electrospun membrane produced from a material such as PCL has been shown to have the required biomechanical properties to support fibrous cell sheets used in the regeneration of soft-hard tissue interfaces such as that between periodontal ligament (PDL) and dentine (Costa et al., 2014; Dan et al., 2014).

Various approaches have been described for tissue decellularization, including chemical, physical and enzymatic treatments (Badylak, Freytes, & Gilbert, 2009). For a decellularization protocol to be efficient, a combination of the aforementioned approaches is usually required (Gilbert, Sellaro, & Badylak, 2006; Syed et al., 2014). Sodium dodecyl sulfate (SDS), an ionic detergent, and Triton X-100 (t-octylphenoxypolyethoxyethanol), a non-ionic detergent, are widely used in many decellularization protocols for their cell lysis capacity (Weymann et al., 2015; Wu et al., 2015). These approaches have reported favourable outcomes including significant elimination of cellular contents and preservation of extracellular matrix structures (Sadr et al., 2012; Syedain, Bradee, Kren, Taylor, & Tranquillo, 2013; Syedain, Meier, Reimer, & Tranquillo, 2013). However, disadvantages have also been reported, such as destruction and removal of the ground substance (glycosaminoglycans), collagen damage and/or deterioration of the mechanical properties of tissues/constructs (Gilbert et al., 2006), with specific decellularization approaches chosen on the basis of their suitability for a particular tissue engineering application. Given that periodontal ligament cell sheets present specific challenges, it is prudent that the most suitable approach is selected for decellularization of the cell sheet constructs.

To this end, this study aimed to investigate different methods for the decellularization of PCL membrane-supported periodontal ligament fibrous cell sheets under both stationary and dynamic fluid conditions, in order to identify the most efficient technique for the removal of cellular contents, which at the same time maximizes extracellular matrix integrity and growth factor retention.

## 2. Materials and methods

### 2.1. Membrane fabrication via melt electrospinning writing

Customized membranes were fabricated using medical grade polycaprolactone (mPCL, Purasorb PC 12, Corbion-Purac) via melt electrospinning direct writing (Brown, Dalton, & Hutmacher, 2011). The polymer was melt electrospun at a temperature of 100 °C, a feed rate of 20 μL/hr, a voltage of 10 kV and a spinneret collector distance of 2 cm. The translational speed of the collector was set at 250 mm/min in order to obtain straight fibers and a square wave pattern was utilized for fabricating a scaffold composed of alternating series of layers oriented at 90°. The membranes were sectioned into 5 mm discs. In order to increase their hydrophilicity, the melt electrospun membranes were etched with 2 M NaOH for 30 min at 37 °C followed by 5 rinses in ultrapure water. The membranes were sterilized by exposure to 70% ethanol for 30 min followed by evaporation under the cell culture hood with another 30 min of UV irradiation. These discs were utilized as a support-membrane in order to harvest the cell sheet and facilitate their handling.

### 2.2. Primary human periodontal ligament cells (h-pdl cells) isolation and proliferation

Primary Human periodontal ligament cells (hPDLC) were obtained according to an established protocol, as previously described (Farag et al., 2014; Ivanovski, Li, Haase, & Bartold, 2001). Briefly, after institutional ethics approval (Griffith University Human Ethics Committee) and informed patient consents were obtained, explants were obtained from diced periodontal ligament tissue sourced from the middle 1/3 of extracted healthy teeth from two different donors. The primary cells from each donor were grown separately throughout the whole study without pooling. Cells were grown to confluence and passaged using 0.05% Trypsin and expanded into 175cm2 flasks. Cells between the 3rd and 4th passages were used in this study.

### 2.3. Cell sheet harvesting

For the preparation of the cell sheets, the h-PDLCs were seeded in 24 well plates with a seeding density of $5 \times 10^4$ cells/well. For the first 48 h, the ascorbic acid (catalogue number: A4403 – L-Ascorbic acid, Sigma-Aldrich) concentration was 1 mg/ml in order to enhance extracellular matrix formation (Beacham, Amatangelo, & Cukierman, 2007). The cells were then grown for 19 days in media supplemented with a lower ascorbic acid concentration (100 μg/ml); the media was changed every 48 h. At the end of the 21 days of culture the cells had deposited sufficient ECM (Fig. 1A) in order to enable the handling of the cell sheet. In order to harvest the cell sheet, a PCL melt electrospun membrane was placed in the centre of the well and the borders of the cell sheet were gently detached from the base of the well and folded over the edges of the membrane using sterile tweezers. The resultant cell sheet constructs (CSCs) were placed in expansion media for 24 h with the cell sheets facing upward, in order to allow cell sheet adhesion onto the scaffold.

### 2.4. Decellularization protocols

Various decellularization methods were utilised and these techniques involved either flow perfusion, or static conditions with and without the utilisation of DNase.

#### 2.4.1. Static decellularization

The CSCs were decellularized by a static method whereby chemicals were added directly onto the construct in a 24 well plate: