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

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Short Title: Static and dynamic decellularization of fibrous cell sheet constructs

Key Words: tissue engineering, periodontal ligament, cell sheet, decellularization, melt electrospinning, polycaprolactone

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Highlights

- Cell sheets constructs were prepared using human PDL placed onto PCL membranes.
- Constructs were decellularized under static/perfusion conditions.
- Collagen and growth factors quantification, immunostaining and SEM were performed.
- No significant differences between different decellularization methods in DNA removal after DNase treatment.
- NH4OH/Triton X-100 and DNase solution was the most efficient method.

Abstract

Objectives: Decellularization aims to harness the regenerative properties of native extracellular matrix and immunogenic cellular material. 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) membrane that reinforced cell sheets during various decellularization protocols. Cell sheet constructs (CSCs) were decellularized under static/perfusion conditions using a) 20mM 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 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 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 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 sheets constructs 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 (A, 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 extra cellular 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 (T. D. Brown, Dalton, & Hutmacher, 2011). The polymer was melt electrospun at a temperature of 100ºC, a feed rate of 20µL/hr, a voltage of 10kV and a spinneret collector distance of 2cm. The translational speed of the collector was set at 250mm/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 5mm discs. In order to increase their hydrophilicity, the melt electrospun membranes were etched with 2M NaOH for 30 minutes at 37ºC followed by 5 rinses in ultrapure water. The membranes were sterilized by exposure to 70% ethanol for 30 minutes followed by evaporation under the cell culture hood with another 30 minutes 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 $5x10^4$ cells/well. For the first 48 hr, the ascorbic acid (catalogue number: A4403 - L-Ascorbic acid, Sigma-Aldrich) concentration was 1000µg/mL 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 (100µg/mL); the media was changed every 48 hrs. At the end of the 21 days of culture the cells had deposited sufficient ECM (Figure 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 resultants cell sheet constructs (CSCs) were placed in expansion media for 24 hours with the cell sheets facing upward for allowing 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.

1. *Static decellularization:*

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

a) Ammonium Hydroxide (NH_4OH) /Triton X-100

The CSCs were rinsed once with PBS and incubated into 20mM NH_4OH (320145 SIGMA-ALDRICH) with 0.5% v/v Triton X-100 (1ml solution per scaffold, 93443 SIGMA) for 30 minutes at 37^{o}C. Thereafter, the decellularizing solutions were replenished and another 30 minutes incubation was performed prior to rinsing the scaffold three times using PBS at 37^{o}C. This method was originally adopted from the technique used by Beacham et al. (Beacham et al., 2007).

b) Ammonium Hydroxide (NH_4OH)/Triton X-100 + DNase

The CSCs were decellularized using 20mM NH4OH with 0.5% v/v Triton X100 (1ml solution per scaffold) for a total of 60 minutes at 37^{o}C as described above. This was followed by immersion in 1ml DNase I solution (100U/ml, Catalog number: 18047-019 Invitrogen) in

CaCl2 (0.9mM) and MgCl2 (0.5mM) in sterile PBS at 37°C for another 60 minutes before a final rinsing step using PBS at 37°C.

c) Sodium dodecyl sulfate (SDS, 0.2% v/v, catalogue number: 05030 SIGMA)

The CSCs were rinsed with PBS and incubated into SDS (0.2% v/v in 1ml solution per scaffold) for 30 minutes at 37°C. Thereafter, the decellularizing solutions were replenished and another 30 minutes incubation was performed prior to rinsing the scaffold using PBS at 37°C [34].

d) Sodium dodecyl sulfate (SDS, 0.2% v/v) +DNase

The scaffolds were decellularized using SDS (0.2% v/v in 1ml solution per scaffold) for a total of 60 minutes at 37°C, as described above. This was followed by a DNase step whereby the CSCs where immersed in 1ml DNase solution (100U/ml) for another 60 minutes and incubated at 37°C before a final rinsing step using PBS at 37°C.

All samples were then rinsed gently three times using ultrapure sterile water, then kept overnight in PBS at 4°C.

2. Perfusion Decellularization

A perfusion system bioreactor was designed in house (Figure 2A & 2B), and consisted of a series of spacers to separate the constructs, as well as silicon tubes, 0.2µm filters and an infusion/withdrawal syringe pump. The chambers and its components were designed with CAD software and additive manufactured using an inkjet 3D printer (Objet30 *Pro* Desktop, Stratasys) with 16 microns deposited layers of an acrylic resin (Verowhite Plus 835, Stratasys). Dipping the chambers in acetone resulted in a water tight enclosure. An infusion/withdrawal syringe pump was used to perfuse the decellularization solutions through the scaffolds, hence allowing for a homogeneous decellularization. Similarly to the static decellularization, chemicals or detergents were utilized using the perfusion system. An additional methodology involving freezing and thawing was also assessed under perfusion.

a) Perfusion + NH_4OH/Triton X-100

The CSCs were rinsed once with warm PBS at 37°C, and then placed in the decellularization chambers with a maximum of 11 constructs per chamber. The CSCs were perfused in 30mL of 20mM NH_4OH solution with 0.5% v/v Triton X-100. Bi-directional perfusion of the constructs

was performed for 60 minutes at a rate of 1000mL/hr with a flow inversion every 50 seconds, followed by a final bidirectional perfusion in 30mL sterile water at 37°C for another 60 minutes.

b) Perfusion + NH_4OH/Triton X-100 + DNase

The decellularization was performed as described above with an added DNase step involving perfusion in 30mL of DNase I solution (100U/mL, Invitrogen) in CaCl2 (0.9mM) and $MgCl_2$ (0.5mM) in sterile PBS at 37°C for 60 minutes and finally perfused with sterile water at 37°C for another 60 minutes (Farag et al., 2014).

c) Perfusion +Sodium dodecyl sulfate (SDS, 0.2% v/v)

The same method as described in the previous section (a) with a similar bi-directional perfusion pattern.

d) Perfusion + Sodium dodecyl sulfate (SDS, 0.2% v/v) + DNase

Here again, a DNase step was performed (described in section b) prior to a final rinsing step with sterile water at 37°C for another 60 minutes under perfusion.

e) Thermal freezing/thawing cycles (F/T)

This method was originally developed by Sadr et al. (Sadr et al., 2012), and was implemented in the present study with minor modifications. The CSCs were initially rinsed with warm PBS. They were placed into 1.5ml cryotubes (Thermo Scientific™) and three successive cycles of freezing/thawing (F/T) were subsequently performed. The freezing/thawing step consisted in immersing the cryotubes into liquid nitrogen for 5 minutes, and then transferring them to a 37°C water bath for 5 minutes. After each step of F/T, the CSCs were rinsed for one minute using warm ultrapure water to hypotonically lyse remaining cells. Finally, scaffolds were bidirectionally perfused for 60 minutes in PBS at 37°C.

e) Thermal freezing/thawing cycles (F/T) + DNase

Perfusion with DNase was added as an extra step prior to final rinsing via PBS perfusion, as a modification to the original Sadr et al. technique outlined.

The CSCs from all experimental methods were kept in PBS overnight at 4^{o}C prior to subsequent evaluation.

2.5. Confocal imaging of cell sheets

The CSCs were immunostained using monoclonal antibodies against human Collagen I (Catalogue #: 63170 MP Biomedicals) to visualize, assess and compare the extracellular matrix and cell sheet integrity of the different decellularization protocols. 4', 6-diamidino-2-phenylindole (DAPI, 5µg/ml) and Phalloidin – tetramethylrhodamine B isothiocyanate conjugate (Phalloidin-TRITC, 0.8U/ml, life technologies, Invitrogen) were utilized to stain the nuclei and the actin fibres respectively. Briefly, samples were fixed with a 4% paraformaldehyde solution at pH7.4 (Sigma-Aldrich, Australia) in phosphate buffer saline (PBS) for 20 minutes and thereafter rinsed with PBS. The cells were then permeabilised for 5 minutes in Triton X-100 (0.2%) in PBS followed by two rinses in PBS. The samples were then incubated for 10 minutes in 1% bovine serum albumin (BSA, Sigma-Aldrich, Australia) in PBS. Primary monoclonal isotype mouse IgG antibody against human Collagen I was diluted in BSA/PBS (1%) with a dilution ratio of 1:200. The antibody solution was added onto the samples and incubated for 45 minutes at room temperature. The samples were rinsed with PBS and incubated in BSA (1%) in PBS containing the secondary Alexa 633 goat anti-mouse antibody (5µg/ml, Alexa Fluor, A-21126 Invitrogen), DAPI at 5µg/ml) and Phalloidin-TRITC at 0.8U/ml in dark for another 45 minutes. Controls for non-specific staining were obtained by omitting the primary antibody. Confocal imaging was undertaken with a Nikon Eclipse microscope (Nikon Eclipse 50iPOL, QUT Central Analytical Research Facility).

2.6. Scanning electron microscopy of cell sheets

Fresh and decellularized CSCs were fixed in 3% Glutaraldehyde for 1 day, and then samples were gradually dehydrated in Ethanol concentrations of 60-100% for 20 minutes in each step, then a post-fixation step with osmium tetroxide (Sigma-Aldrich, Australia) was performed for 60 minutes. The samples were left to dry overnight, then mounted on adhesive stubs with cell sheets facing upwards, gold coated under vacuum for 3 minutes then Imaged using FEI Quanta 200 SEM.

2.7. Quantification of DNA

DNA content was measured in both fresh and decellularized CSCs. The specimens were transferred to a new sterile 24 well plate, sealed with parafilm and placed at -80ºC for 48 hours. The samples were thawed at room temperature then transferred to Eppendorf tubes containing 300µl of Proteinase K solution (life technologies- Invitrogen) diluted in PBE 1:50 for a final concentration of 0.5mg/ml, then placed at 65ºC for 8 hours in a thermo-mixer (Eppendorf thermomixer-BioExpress). Samples were diluted 1:20 in PBE; a standard curve was prepared with λ DNA standard (Quant – iT PicoGreen) using the dilution series method according to the manufacturer's instructions. Test and standard solutions (100µL) were plated in triplicate in a black 96 well plate (MicroWell –NUNC, Thermo Fisher Scientific). Thereafter, 100uL of PicoGreen dye solution prepared according to the manufacturer's instructions was added to each well and incubated for 10 minutes in the dark. A fluorescence plate reader (BMG PolarStar, Ottenberg, Germany) with excitation 480nm and emission wavelengths of 480nm and 520nm respectively was used to measure the fluorescence. A standard curve with known DNA concentrations was used to calculate the final DNA content in each sample.

2.8. Collagen quantification:

Collagen content in the CSCs was measured in fresh and decellularized samples using a Hydroxyproline assay kit (Chondrex, Inc. - catalog#6017) according to the manufacturer's instructions. Briefly, the samples were placed in sterile 1.5ml Eppendorf tubes and 100µl of distilled water was added. The samples were sonicated for 30 seconds in cycles of 3 seconds pulse and 1 second rest with an amplitude of 50%. 100µL of 10N HCL was then added and incubated for 24 hours at 120°C. The samples were cooled down, transferred to micro centrifuge tubes and subsequently centrifuged at 10,000 rpm for 3 minutes. A standard curve was prepared from known collagen concentration solutions.

The samples were used undiluted. 10µL of 10X Chloramine T solution and 90µL of Chloramine T dilution buffer were added for each sample in a 96 well plate, where samples and standards were plated in duplicates, then incubated for 20 minutes. 50µL of 2X Dimethylaminobenzaldehyde (DMAB) solution added to 50µL of DMAB dilution buffer was then added to each well, incubated for 30 minutes at 60°C, and the optic density was evaluated using a plate reader (BMG PolarStar®) at a wavelength of 530-560 nm. Hydroxyproline levels

(µg/mL) were determined using a standard curve prepared from reagents included in the assay kit, according to the manufacturer's instructions.

2.9. Growth factor Bioplex assay

Basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and Hepatocyte growth factor (HGF) levels in fresh and decellularized CSC samples were quantified using multiplex immunoassays (Bio-Plex Pro^{TM}-Bio-Rad). Growth factor extraction was carried out as previously described (Farag et al., 2014; Reichert et al., 2010; Thibaudeau et al., 2014) whereby 300µl of 2M NaCl in 20mM HEPES with EDTA protease inhibitor cocktail (Roche complete mini, Roche Applied Science, Indianapolis, IN) was added to each sample then incubated for 60 minutes at room temperature on an orbital shaker. Solutions from each sample were collected in 1.5ml Eppendorf tube and centrifuged at 2000 rpm for 5 minutes then kept in -80°C till the time of the analysis. Assay for growth factors was done using a Bio-plex system (Bio-Plex Pro^{TM}-Bio-Rad) according to instruction manual provided by the manufacturer. Briefly, samples were placed on ice and gradually thawed to room temperature. Bio-Plex Pro™ Reagent Kit with Flat Bottom Plate (#171304070M) was used for the analysis. 50µL of magnetic beads were added to selected wells of a 96 well plate provided by the manufacturer and washed twice with 100µl wash buffer using a handheld Magnetic Washer (Bio-Plex® #171020100). Quantitative analysis was carried out using standards prepared in a series of dilutions (group I human cytokines (#171-D50001) for basic fibroblast growth factor (bFGF) and vascular endothelial factor (VEGF) and group II human cytokines (#171-D60001) for hepatocyte growth factor (HGF)). Briefly, 50µL of standards, blank, and samples (undiluted) were plated in duplicate and incubated at room temperature with shaking at 850 rpm for 60 minutes. The plate was then washed three times with 100µL wash buffer, and 25µL of detection antibody was added to the wells and incubated for 30 minutes at room temperature protected from light on a shaker at 850 rpm. After being washed again with wash buffer three times, 50µL of Streptavidin-PE was added to each well, left for 10 minutes at room temperature protected from light on a shaker, followed by a final washing step. Finally, 125µL of assay buffer was added for 30 seconds and the plate was read using a Bio-Plex suspension array system (Bio-Plex® 200 System) and concentrations were obtained in picograms.

2.10. Statistical analysis

Results were expressed as mean + standard deviation from at least five biological replicates and one-way ANOVA test was used to analyse the data. Tukey's multiple comparison post hoc test was used to determine the significance. The significance level for the statistical analysis was set at $p < 0.05$.

3. RESULTS

3.1. DNA removal and DNase effect on decellularization

Static decellularization was found to be generally more efficient in the elimination of DNA contents of the CSCs when compared to the perfusion technique (Figure 3a). Treatment with SDS resulted in 75% and 40% of DNA removal using static and perfusion decellularization respectively. The NH_4OH/Triton X-100 protocol eliminated only 53% of DNA using the static method, while DNA elimination was almost negligible with the use of perfusion. DNA removal was significantly improved when DNase was added as an extra step after the initial decellularization. DNA was not detected in both the static and perfusion techniques utilizing the SDS-DNase combination, thus indicating complete DNA removal. NH_4OH/Triton X-100 combined with DNase eliminated all DNA content using static decellularization and up to 97% with the perfusion method. The freezing thawing (F/T) technique did not achieve adequate DNA elimination (approx. 31% of DNA content was removed) but with the addition of a DNase step, DNA elimination was significantly improved to around 91%.

3.2. Structural preservation of decellularized sheets

Structural integrity of the cell-containing CSC extracellular matrix (Figure 1 B-E) and following the different decellularization protocols (Figure 4&5) was assessed using Scanning Electron Microscopy (SEM) and by immunostaining against human collagen type I that was visualized using confocal microscopy. CSCs treated with SDS with or without DNase were superior to all other techniques in terms of elimination of cellular and nuclear contents as seen by the lack of nuclear DAPI staining on the confocal imaging (Figures 4G, 4J, 5G & 5J). However, SDS was noticeably destructive to the collagen fiber architecture when compared to both the NH4OH/Triton X-100 and Freezing–Thawing decellularization techniques. Disruption was manifested as alteration in the fibrous network structure of the cell sheet by fusion or coarsening of collagen fibers and/or loss of the fine fibrils normally seen in the

untreated cell sheets (Figure 4I&5I). Structural integrity was even more negatively affected in samples treated by the SDS–DNase combination, with more significant alteration in the fibrous structure of the extracellular matrix as demonstrated by SEM (Figure 4L&5L). Conversely, the NH4OH/Triton X-100 decellularization protocol preserved the extracellular matrix to a greater extent as demonstrated by SEM, but abundant cellular and nuclear debris was present (Figure 4C&5C). However, the addition of a DNase step retained the structural integrity of the CSC collagen fibers with significantly enhanced removal of cell debris as demonstrated at higher magnifications by SEM (Figure 4F&5F). The Freezing/Thawing technique (F/T) appeared to preserve the collagen fibrous structure of the CSCs as seen in the immunostaining and SEM imaging, however significant cellular debris remained (Figure 5M&5O), which was significantly reduced after the addition of a DNase step (Figure 5P&5R). F/T with or without the addition of DNase appeared to result in partial disruption of the CSC extracellular matrix (Figure 5M&5P). Generally, cellular and nuclear contents were greatly reduced or absent in samples treated with DNase, as seen by confocal microscopy (Figures 4D, 4J, 5D, 5J & 5P) and SEM (Figures 4F, 4L, 5F, L&R).

3.3. Collagen content preservation

Generally, the various decellularization techniques did not seem to alter the collagen content in the CSCs with the exception of the static SDS treatment which reduced the collagen content by approximately 35%. NH_4OH/Triton X100 and F/T cycles preserved most of the collagen content in the cell sheets. Also, DNase enzymatic treatment did not seem to have any significant effect on collagen preservation (Figure 3B).

3.4. Growth factors retention

Absolute quantities of all growth factors were significantly reduced in the decellularized CSCs irrespective of the method of decellularization. Static and perfusion methods showed comparable results in the percentage of growth factor (FGF, VEGF and HGF) retention in the decellularized sheets. Around 10% of the original growth factors (in a fresh cell sheet) were retained when NH4OH/Triton X-100 was used under either static or perfusion conditions. Similar values were obtained with the F/T decellularization method. Retained quantities of FGF and HGF were either very low or undetectable in cell sheets decellularized using SDS. DNase treatment did not appear to affect the amounts of retained growth factors in the CSCs irrespective of the decellularization protocol with which it was combined (Figures 3C, 3D & 3E).

4. Discussion

Decellularization is a strategy that aims to harness the regenerative properties of native extracellular matrix while reducing potentially immunogenic cellular components. Cell sheet technology is a tissue engineering approach which has been recently used in the clinic to facilitate the *in vivo* delivery of cells together with an intact extracellular matrix (Egami, Haraguchi, Shimizu, Yamato, & Okano, 2014; Ohki et al., 2012; Sawa et al., 2012). Scaffold cell sheet constructs may be particularly useful for regenerating soft-hard tissue interfaces, such as those encountered in the periodontium, and combined with appropriate decellularization methods have the potential to be developed as "off-the-shelf" constructs for clinical use. In order to identify the best decellularization method that efficiently removes cellular material and maintains extracellular matrix integrity and growth factor retention, this study investigated the influence of various protocols on the structural integrity and growth factor retention in decellularized periodontal ligament fibrous cell sheets.

A well-recognized challenge of using cell sheets is the difficulty in handling, delivering and stabilizing an intact cell sheet (Flores, Yashiro, et al., 2008; Ishikawa et al., 2009). In order to improve cell sheet handling, we previously utilized a thin membrane made of medical grade electro-spun polycaprolactone (PCL) as a carrier scaffold that provided appropriate biomechanical support to facilitate periodontal regeneration (Dan et al., 2014), and this membrane was also utilized in the current study. Given that the handling challenges are exacerbated during decellularization because of the additional manipulation that is required, a support structure, such as the PCL membrane used in this study, should be considered an absolute requirement for cell sheet stabilization during the various decellularization protocols.

In this study, both the static and perfusion techniques were efficient in cellular and nuclear content removal, as measured by the amount of remaining DNA. The rationale for DNA removal is to avoid or at least minimise a possible immune response upon *in vivo* transplantation [37]. Although the static method was relatively superior in terms of DNA removal compared with the perfusion technique, neither technique achieved favourable DNA removal without the use of DNase I. Therefore, the use of DNase can be considered essential for effective DNA removal.

It was also noted that collagen integrity and content of the cell sheets was not different between the static and perfusion techniques. Further, there was no difference in growth factors retention between the static and perfusion methods when the same chemicals were used. Therefore, the efficiency of the decellularization and extracellular matrix structural and biological integrity appears to depend mainly on the nature of the chemicals, detergents and/or enzymes used, and to a lesser extent by the fluid mechanics during the decellularization process. However, it should be considered that the perfusion technique is more practical, technically less complicated and less time consuming when compared to the static technique, which would be important in the context of future upscaling for commercial applications. This is also the likely reason that the perfusion method appears to be favoured by recent studies utilizing decellularization of tissue engineered constructs (Bao et al., 2011; Syed et al., 2014).

The utilization of chemicals and detergents, with or without enzymes, plays a key role in the efficiency of decellularization. In this study, it was shown that more than 50% and 75% of DNA content were removed with SDS using static and perfusion decellularization protocols respectively, and almost complete DNA elimination (up to 99%) was achieved when enzymatic treatment using DNase was added. However, this came at the expense of growth factor retention. Indeed, growth factor concentrations were not only significantly decreased compared to other methods, but were also reduced to the extent that FGF could not be detected in the decellularized sheets. These results appear to contradict the findings of a recent study, which reported that dermal fibroblast cell sheets treated with SDS retained considerable amounts of FGF and VEGF (Xing et al., 2015). The differences might be attributed to that study's longer culture period and shorter period of decellularization, or the higher SDS concentration used in the present study. However, it must be noted that the decellularization conditions that resulted in the reported retention of growth factors following SDS treatment also resulted in the suboptimal removal of only between 45% (for low SDS 0.05 wt%) and 90% (for high SDS 0.5%) of DNA, as well as altered collagen structure as confirmed via immunostaining and SEM. This was consistent with a reported altered pattern of immunofluorescent staining for collagen type I and III in porcine bladders treated with SDS (A. L. Brown et al., 2005). These observations can be attributed to the denaturing effect of SDS on proteins of the extracellular matrix, and/or removal of other ECM components. It is also noteworthy that decellularized constructs treated with SDS were difficult to handle and decellularized sheets tended to detach from the PCL scaffolds due to loss of ECM integrity. This is consistent with the findings of a recent study (Faulk et al., 2014) which evaluated the effect of different detergents on the

basement membrane complex (BMC) and showed that SDS treatment had a detrimental effect on BMC integrity.

A thermal method for decellularization in the form of freeze-thaw (F/T) cycling was also evaluated. This approach has been favoured in several recent studies as it does not involve utilization of detergents and other potentially harmful chemicals, while achieving DNA removal of up to 96% with favourable preservation of the extracellular matrix integrity (Burk et al., 2014; Nonaka et al., 2014; Sadr et al., 2012). This method was found to be less efficient in decellularization of cell sheets in the current study unless an extra DNase treatment step was added. It is also noteworthy that the F/T method was more time consuming when compared to other techniques. Although collagen structure and content were preserved and growth factor retention was favourable, the gross overall integrity of the cell sheet was negatively affected by the F/T cycles as observed in both immunostaining and SEM imaging (Figure 5M, N, P &Q).

The combination of NH_4OH/Triton X-100 and DNase was found to be the most efficient method of ECM structural preservation and DNA elimination irrespective of the whether the decellularization was carried out under static or perfusion conditions. Consistent with previous experiences with this method, DNA removal was efficient (up to 92%) without sacrificing the structural integrity of the ECM or the retention of the native growth factors in the decellularized sheets (Farag et al., 2014). This is consistent with the findings of a recent study which compared the efficacy of different protocols for preparation of extracellular matrix scaffolds derived from three-dimensional cell culture, and concluded that the combined NH_4OH and Triton X-100 treatment performed the best in terms of the removal of cellular components from the complexes (Lu, Hoshiba, Kawazoe, & Chen, 2012).

This study assessed different protocols for the decellularization of periodontal ligament cell sheets, and showed that the combination of NH_4OH and Triton X-100 together with a DNase treatment step was the most efficient method for DNA removal and preservation of extracellular matrix integrity and growth factors retention, irrespective of whether a static or perfusion approach was used. The perfusion protocol may have the added advantage for automatization and throughput upscalability that may facilitate future commercialization. Since periodontal ligament cells are widely recognized to exhibit both inter- and intra- population heterogeneity (Ivanovski et al, 2001; Lekic et al, 1997), an important consideration for future commercialization is the need to have a standardized cell source for preparing the

decellularized cell sheets. Indeed, future research should aim at characterizing the correlation between the nature of individual primary human periodontal ligament cell populations and the bioactivity of the resultant decellularized cell sheet constructs.

Conflict of interest

The authors have declared that there is no conflict of interest.

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Figure legends

Figure 1. Light microscopy, confocal microscopy and SEM of mature PDL cell sheet prior to decellularization. A, light microscopy image of mature cell sheet. B, Collagen I immunostaining (White) of the fresh cell sheet, DAPI staining of nuclei (Blue) and phalloidin staining of the actin filaments (Red). C-E, SEM showing the cells and associated fibrous network at different magnifications.

Figure 2. Decellularization perfusion system. A, The perfusion system composed of the bi-directional syringe pump connected to the perfusion chambers through silicon tubes. B, decellularization chamber components comprising of the inner carrier and the housing chambers. The chamber was CAD designed and 3D printed using photocurable material.

Figure 3. DNA, collagen and retained growth factor quantification in decellularized human periodontal ligament cell sheets. Results having the same letter are not significantly different ($P \leq 0.05$). A, Remnant DNA in the decellularized sheets was quantified by PicoGreen assay. B, Collagen content was measured using a hydroxyproline release assay. C-E, Residual growth factors detected in the fibrous cell sheets using a Bio-plex assay. NH_4OH/Triton X-100 + DNase using perfusion was superior in DNA removal, preservation of collagen and growth factor retention.

Figure 4. Confocal imaging and SEM of static decellularized cell sheets. A; D; G; J, Collagen type I immunostaining (White), DAPI staining of nuclei (Blue) and phalloidin staining of the actin filaments (Red). B&C; E&F; H&I; K&L, SEM showing the fibrous network after static decellularization at different magnifications.

Figure 5. Confocal imaging and SEM of perfusion decellularized cell sheets. A; D; G; J; M; P, Collagen I immunostaining (White), DAPI staining of nuclei (Blue) and phalloidin staining of the actin filaments (Red). B&C; E&F; H&I; K&L; N&O; Q&R, SEM showing the fibrous network after perfusion decellularization at different magnifications.