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Decellularization of Human Lip

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Abstract

Introduction: Perioral tissue loss commonly requires surgical reconstruction. Autologous tissue transfer result in poor functional and aesthetic outcomes and allogenous transplantation of tissue requires lifelong immunosuppression. There is a clinical need for a cell scaffold, which could be seeded with the patients own cells to create an immunogenically inert perioral tissue replacement. Decellularized human lip may provide the answer.

Methodology: To the authors knowledge this was the first time human lip has been decellularized. Four existing protocols shown to be successful at removing cells from either muscle or dermis were used to decellularize human lip in an attempt to identify an optimal protocol.

Results: Three of the four protocols proved to be successful at achieving decellularization of the lip, as histological investigation of these samples showed complete loss of cellular structures for the entire construct. A non-detergent based protocol using osmotic shock and enzymatic processes best preserved the extracellular matrix. It was able to maintain the micro-architecture of collagen and elastin, and retain important signaling molecules such as glycosaminoglycans.

Conclusion: This decellularized scaffold developed here may be the first step towards an exciting new treatment for perioral tissue loss.

Keywords: Cell scaffold; Dermis; Muscle; Regenerative medicine; Lip; Facial surgery; Human and decellularization

Introduction

The human lip is a complex structure, essential for communication and in the function of the oral sphincter during swallowing and imbibition. During surgical reconstruction of the lip, restoring the cosmetic appearance, as well as the functional integrity is important. Even a millimetre step in the alignment is noticeable at conversational distances, having a significant impact on self-esteem and quality of life [1].

Perioral tissue loss has many causes including neoplasm, trauma and congenital deformities. Conventional techniques for perioral reconstruction such as direct closure, local flaps and distant free flaps, are adequate for small areas of tissue loss but for larger defects they often give unsatisfactory results [2-5]. Surgeons acknowledge that once lost, the central part of the upper lip cannot be fully restored by any conventional surgical technique [6]. Allogenous face transplantation provides an alternative treatment option [7], however it has its drawbacks. Finding suitable donors can be a long process and recipients are required to take lifelong immunosuppression, which causes significant morbidity [8].

A tissue engineered construct capable of mimicking the functional and aesthetic traits of the human lip, whilst also being immunogenically compatible with the host, would provide a major breakthrough in facial surgery and benefit a vast number of patients clinically. Decellularization of human lip has the potential to create such a scaffold.

Decellularized natural scaffolds are already used clinically for the reconstruction of dermis, cardiac valves and the urinary bladder [9-12]. Promising work is also underway to develop acellular scaffolds for dynamic organs such as the heart and skeletal muscle [13-16]. To the authors knowledge no attempts have been made to date to decellularize the human lip. Decellularization of the entire lip would require a protocol capable of removing cells from skeletal muscle and the surrounding soft tissue including the dermis of the skin, vermillion and mucosa.

The bulk of the lip is made up of Orbicularis oris a complex skeletal muscle. It muscle fibres have a unique orientation and are suspended from the surrounding muscles without any bony attachments. Decellularized muscle seeded with myoblasts has been shown to be capable of generating a contractile force on electrical stimulation [17]. However decellularization of muscle has so far been limited to very small muscles or fragments of muscle and in vivo successes are limited to small animal models [18-22].

There are already a number of commercially available acellular dermal scaffolds. These products have found many uses in clinical applications such as burns dressing [9], abdominal wall repair, breast surgery facial reconstruction and intra-oral mucosa reconstruction [23-28]. Decellularization of dermis first involve the removal of the epidermis, which can be achieved by using a hypertonic saline solution or an enzymatic method [10,29,30]. The remaining dermis is decellularized using either detergent or enzymatic methods. The

resulting scaffolds have been repeatedly shown to be non-immunogenic and capable of supporting cell proliferation.

Methodology

Overview of Study Design

As this was the first attempt at decellularization of the lip, samples were subject to four existing protocols, in an attempt to identify an optimal method for decellularizing this complex structure.

Two protocols were based on existing research into decellularization of skeletal muscle: a protocol by Wang et al. which has been used to successful decellularization porcine rectus abdominis muscles and a protocol by Gillies et al. which avoids the use of detergents relying instead on osmotic shock and enzymatic methods only for removal of cells from the tibialis anterior muscle in mice [14,16].

The remaining two protocols were based on existing research into the decellularization of dermis: a protocol by Livesey et al. and Walter et al. used on porcine skin and cadaveric skin respectively and a protocol based on Chen et al. used to decellularize porcine dermis [10,29,30].

The protocols were all initially designed for smaller structures then the slices of human lip used in this study, therefore if after the first cycle the protocol had not achieved complete decellularization of the tissue, then a second cycle of the protocol was be carried out.

Tissue Harvest

All experiments were carried out in accordance with the Human Tissue Act (2004). Samples were obtained from fresh frozen humans cadavers and stored at minus 80°C. The tissue was thawed at room temperature and hair from the upper lip was removed using Veet® hair removal cream. The upper lip was then harvested along its anatomical surface markings, from 4 individuals (3 male and 1 female), with an age range between 63 and 75. The lips were sliced into 5 mm thick cross-sectional slices, transverse to the muscle fibres using a sharp scalpel. The slices used for the experiment were taken from the portion of the lip immediately lateral to the philtral ridge (within 2 cm of this landmark). These slices were then divided into 5 groups (A, B, C, D and control). Groups A, B, C and D were each subject to a separate decellularization protocol, detailed below. The control group was frozen at minus 20°C until processing.

All further steps were carried out at room temperature and under agitation unless otherwise stated. After decellularization the sampled were stored at minus 20cC prior to analysis.

Protocol A

Samples were frozen at minus 80°C and thawed rapidly at 37°C for 3 cycles and washed in distilled water for 2 days. These samples were treated with 0.5M sodium chloride for 4 hours, followed by 1 M sodium chloride for 4 hours, and washed in distilled overnight. The hypotonic/hypertonic solution steps were repeated once. The samples were treated with 0.25% trypsin/EDTA at 37°C for 2 hours; washed in distilled water for 1 hour; treated with 1% Triton X-100 for 5 days (one change every day); DNase at 37°C for 3 hours; washed in distilled water for 2 days and rinsed in PBS for 1 day [16].

Protocol B

Samples were thawed rapidly at 37°C and incubated in 50nM Iatunculin B in high-glucose Dulbecco's modified Eagle's medium for 2 hours at 37°C; 0.6M potassium chloride for 2 hours; 1.0M potassium iodide for 2 hours and washed in distilled water overnight, before the potassium chloride and potassium iodide steps were repeated. Tissue was incubated with DNase I (1kU/mL) for 2 hours and washed in distilled water for 2 days (daily water change). Samples were washed twice with distilled water for 15 minutes between each step [14]. After one cycle it was evident on histological examination that decellularization was not achieved. Therefore the protocol was repeated.

Protocol C

Samples were frozen at minus 80°C and thawed rapidly at 37°C in saline. They were then treated with 1M sodium chloride for 24 hours at 37°C; 0.5% SDS for 1 hour and washed extensively with PBS. Samples were also washed twice with distilled water for 15mins each between each step. After one cycle it was evident on histological examination that decellularization was not achieved. Therefore the protocol was repeated.

Protocol D

Samples thawed rapidly at 37°C and incubated with 0.25% trypsin solution for 42 hours with a solution change at 18hours. Samples were then washed with 0.1% sodium dodecyl sulphate (SDS) at room temp for 12 hours, followed by Dipase I 560U/l for 12 hours and SDS 0.1% again for 12 hours. Between each step the samples were washed in two changes of PBS for 15 minutes each [30].

Histology and Fluorescence Studies

Samples were fixed in formalin for 24 hours and paraffin-embedded following standard protocol. The middle one third of each sample was used to produce 5 μm sections manually using a microtome. Samples were stained using standardised protocols for hematoxylin and eosin stain (H&E); millers and picro-sirius red stain; alcian blue with periodic acid–schiff stain; and DAPI for florescence studies.

Results

Removal of Cells

To assess the effectiveness of each decellularization protocol at removing cells, the samples were stained with H&E to reveal any remaining nuclei (Figure 1 and Figure 2). Each tissue type in the lip was looked at in detail to check for remaining cells. No nuclei were visible for samples treated with protocol A and D suggesting complete decellularization had been achieved.

Samples treated with protocol B showed significant reduction in nuclei however a few nuclei were still evident (Figure 3). This may represent satellite cells that are more resistant to decellularization[14]. Dark staining was also visible on the peripheries of the dermis in these samples. For this reason the protocol was repeated and after the second cycle (protocol B2) no nucleic structures were visible in any part of the tissue.

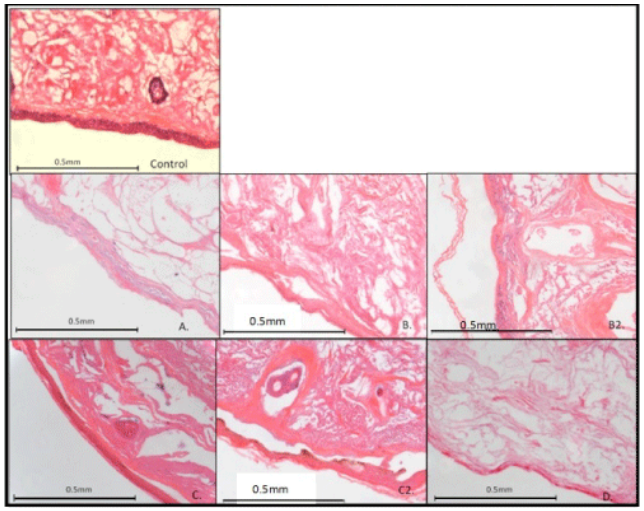

Figure 1: H&E staining of the skin (Original magnification x10): Control-visible nuclei in all skin layers; Protocol A-no visible nuclei; Protocol B-no visible nuclei, however nonspecific haematoxylin staining in epidermis; B2 (second cycle B)-no nuclei; Protocol C-visible nuclei in all layers; Protocol C2 (second cycle C)-nuclei number reduced still visible; Protocol D-No nuclei visible 0.Srmn 0.5mm.

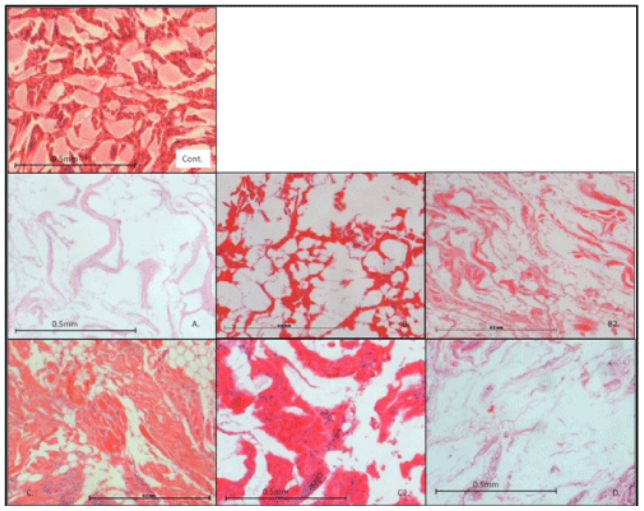

Figure 2: H&E staining of muscle (Original magnification x10) Control-visible nuclei; Protocol A-no visible nuclei; Protocol B-Occasional visible nuclei; B2 (second cycle B)-no nuclei; Protocol C-visible nuclei; Protocol C2 (second cycle C)-visible nuclei; Protocol D-No nuclei visible

These findings were confirmed with DAPI studies (Figure 3 and 4). Samples treated with protocol B2 and D showed no fluoresces in the entire structure. Protocol B showed minor areas of florescence mainly in the periphery but no evidence of discrete nuclei. Interestingly even though no nuclei were present on H&E staining after protocol A, on DAPI staining for nucleic structures there was significant florescence visible throughout the tissue. However, this florescence was not contained within discrete nuclei as demonstrated by higher magnifications images (Figure 3). This may represent incomplete clearance of cellular matter following cell lysis or may represent artefact.

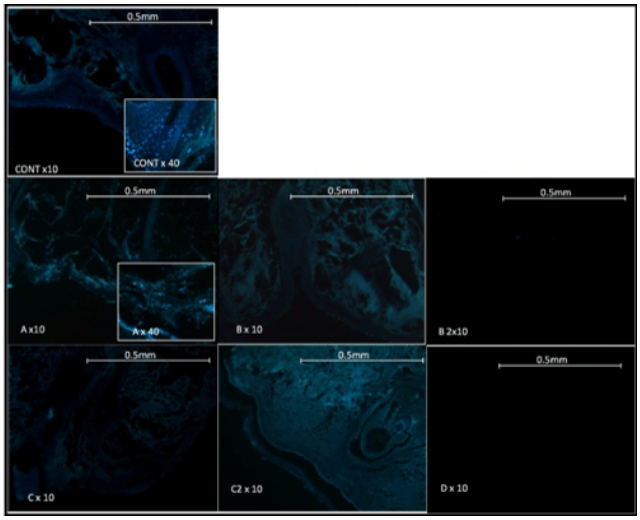

Figure 3: DAPI of skin (main images original magnification x10, insert images x40): Control-visible nuclei, insert demonstrates discrete appearance of nuclei. Protocol A-no discrete nuclei, however non-specific fluorescence throughout tissue; Protocol B-Occasional nuclei; B2 (second cycle B)-no nuclei; Protocol C and C2 (second cycle C)-nuclei visible; Protocol D-No nuclei visible.

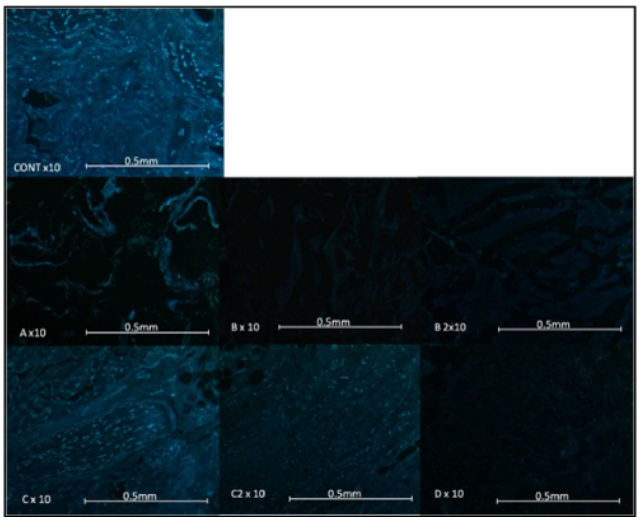

Figure 4: DAPI staining of muscle (Original magnification x10) Control-visible nuclei; Protocol A-no visible nuclei, non-specific fluorescence pattern; Protocol B-Occasional visible nuclei; B2 (second cycle B)-no nuclei; Protocol C-visible nuclei; Protocol C2 (second cycle C)-visible nuclei; Protocol D-No nuclei visible

Nuclei were evident throughout the samples treated with protocol C, even after it was repeated (C2). This protocol was not successful at achieving decellularization.

Decellularized ECM Composition and Structure of Skin

Samples were stained with Millers and Picro-sirius Red stains to assess the microstructure of the ECM. The resulting images show collagen as red, elastin as purple and nuclei as dark blue/black. Looking at the epidermis, protocol A and D were successful at achieving compete removal of this cellular layer. Protocol B showed some disruption to the epidermis and B2 showed further areas of disruption with large portions completely removed (Figure 5). Protocol C and C2 showed complete detachment of the epidermis however as it was not physically removed from the surface the remnants are still visible in the slides.

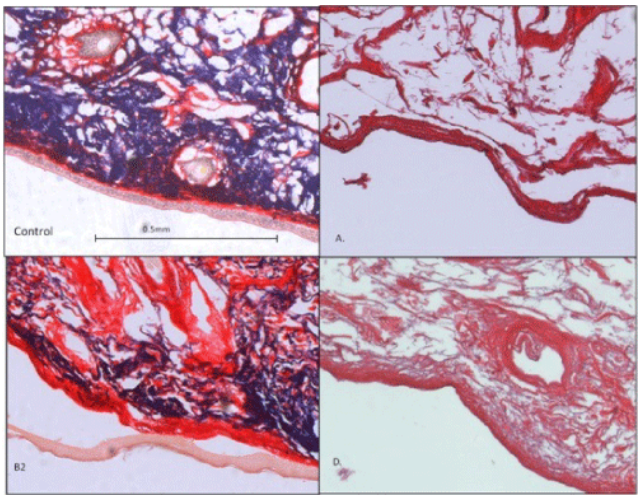

Figure 5: Areas of disruption with large portions completely removed

There was some degree of disruption to the ECM of the dermis and hypodermis with all protocols. Protocol A resulted in almost complete loss of elastin content and disruption collagen microstructure. Protocol D also resulted in significant loss of elastin from the skin, however the collagen content and microstructure appeared better preserved. Protocol B2 maintained much the elastin structure and collagen content was generally well maintained but its microstructure somewhat disrupted. As protocols B, C and C2 did not achieve complete decellularization they will not be discussed further.

Protocol B2 appeared to have a minor effect on GAG content whilst both protocols A and D caused significant removal of GAGs from the skin (Figure 6).

Decellularized ECM Composition and Structure of Mucosa and Vermillion

Protocols A, B, B2 and D resulted in de-epithelialisation of the mucosa. The underlying collagen matrix of their dermis was severely disrupted with protocol A with a significant loss of elastin content. However the microstructure was remained relatively well preserved with protocols B2 and D (Figure 7). The GAG appeared to be most disrupted in the mucosa by protocol D, with less detrimental effects caused by A and B2.

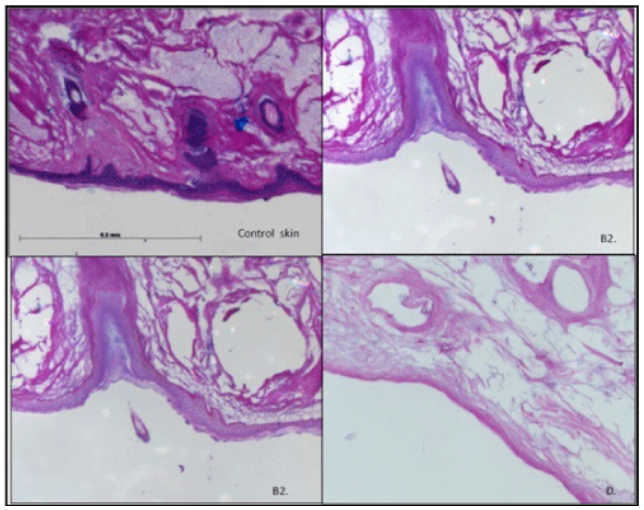

Figure 6: Removal of GAGs from the skin.

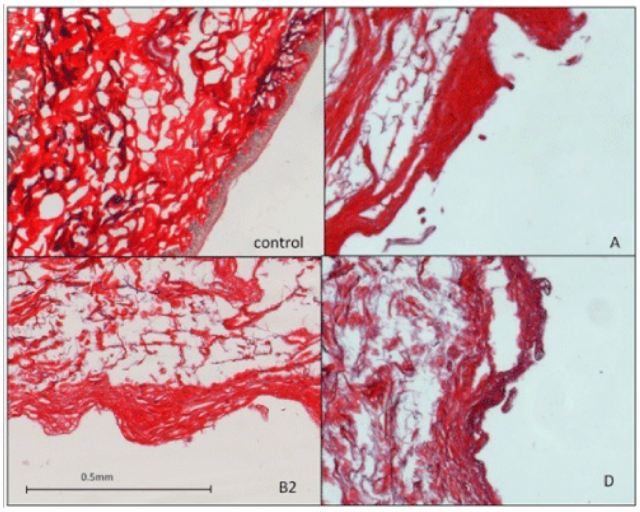

Figure 7: Microstructure well preserved with protocols B2 and D.

Decellularized ECM Composition and Structure of Muscle

The collagen and elastin content and ECM architecture of skeletal muscle was severely affected by protocol A, with large holes in the tissue as demonstrated in Figure 8. There was less disruption to these structures with protocol B2 and D in which the microstructure of the ECM was relatively well maintained. The GAGs again were largely removed by protocol A but better preserved by protocol B2 and D.

Macroscopic Appearance of Tissue

Protocols A and D resulted in expansion of the tissue, loss of shape and a flaccid consistency. Protocol B2 maintained its macroscopic size and shape better than the other two successful protocols (Figure 9).

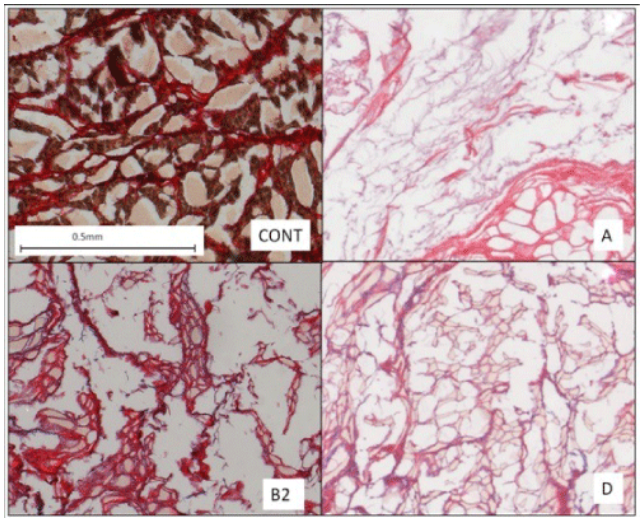

Figure 8: Protocol A, with large holes in the tissue.

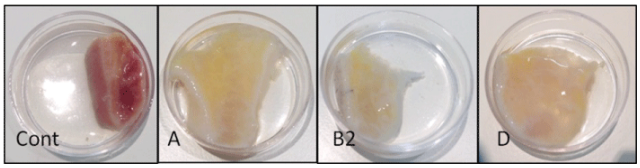

Figure 9: Protocol B2 maintained its macroscopic size and shape.

Summary of Results

Protocol A was successful at achieving complete decellularization of the lip; however it caused severe disruption to the architecture of the ECM, in particular a significant loss of elastin and GAGs, and damage to the collagen architecture, resulting in a flaccid misshaped tissue. Protocol B was not successful at achieving decellularization after one cycle but after repeating the protocol there was no evidence of nucleic structures remaining. The ECM was well preserved, indeed better than any of the other protocols with a good retention of collagen, elastin and GAGs. The macroscopic structure maintained its shape and consistency better the other protocols. Protocol C did not achieve decellularization even after 2 cycles. Finally protocol D achieved complete decellularization, however like protocol A resulted in a significant decrease in elastin and GAGs, a slightly better preserved collagen architecture but macroscopic appearance was again of a flaccid misshaped tissue.

Discussion

The present study describes four decellularization methods for slices of human lip; two protocols based on existing studies for dermis and two on studies for decellularization of skeletal muscle. The effectiveness of each protocol in achieving complete decellularization was assessed, as was the extent of damage to the native ECM, looking in particular at the content and microstructure of collagen, elastin and GAGs. Protocols A and D achieved decellularization after one cycle. Protocol B required 2 cycles to remove all cellular material and protocol C was unsuccessful even after 2 cycles.

Protocol A was based on a study by Wang et al. for the decellularization of slices of porcine rectus abdominis muscle using freeze/thaw, osmotic shock, trypsin and Triton X-100. The protocol appeared to be successful at removing all nucleic structures form the tissue suggesting complete decellularization. However there was evidence of DAPI uptake throughout the remaining ECM, which did not appear to be contained within nuclei. This may be due to incomplete clearance of DNA released from the lysed cells. It could also be due to artefact uptake generated by either the decellularization process or fixation process. To differentiate between these explanations the next step would be to quantitatively measure the DNA content remaining in the tissue using a PicoGreen assay technique [39].

However even if as the lack of nuclei suggests the tissue was indeed decellularized, the remaining ECM was severely disrupted. There was almost a completely loss of elastin from the tissue and large holes visible within the collagen matrix, especially in the ECM of the skeletal muscle. GAG content was also significantly reduced. These finding would suggest that cells cultured on this scaffold would not benefit from optimal mechanical and chemical signals for differentiation and proliferation.Additionally the macroscopic appearance revealed a flaccid misshapen structure, which would make surgical handling of the tissue difficult and impractical for clinical use.

Protocol D based on a process developed by Chen et al. involved removal if epidermis using trypsin, followed by decellularization with trypsin, SDS and Dipase. The resultant acellular scaffold showed no evidence of residual cellular material within the tissue. The epithelium was completely removed. Being the shortest of the three successful protocols makes it appealing for use commercially. However the resultant ECM did show areas of significant disruption, especially within the skeletal muscle. Additionally while the microstructure of collagen was well preserved in the dermis, there was a significant loss of elastin and the macroscopic appearance was again of a flaccid misshapen structure which would not provide practical handling qualities for surgical use.

Protocol B was the most successful overall. It was based on a decellularization process developed by Gillies et al. that avoids the use of harsh detergents and relies instead on osmotic shock and enzymatic processes. After two cycles the protocol produced a decellularized tissue that had a well maintained macroscopic appearance. The ECM of the scaffold was well preserved maintaining much of its collagen, elastin and GAG structure. There were some areas of damaged to the ECM particularly in the skeletal muscle, however out of three successful protocols, protocol B2 had the most well preserved microstructure. Further assessment of its mechanical properties should be carried out with formal mechanical testing to confirm these findings.

After once cycle of protocol B there was almost complete decellularization of the lip but there were a number of scattered nuclei still visible in the muscle. These are likely to represent satellite cells which have a greater resistance to decellularization [14]. This could be confirmed by staining for the satellite cell marker PAX 7 [40]. The reason this protocol did not achieve complete decellularization after one cycle may be simply due to the fact that it was designed for much smaller muscles: tibialis anterior from mice [14] and cricoarytniod muscles from rabbits [33]. However a contributing factor may have

been insufficient volumes of solutions used in this experiment, especially as this protocol relies so heavily on osmotic shock. It is possible that the 5mls of hypotonic solution used became saturated with solutes and was prevented from achieving its maximum effect.

An additional drawback to this protocol is that the epidermis remained attached in small segments of the skin. Complete removal of this water resistant layer will become increasingly important when decellularizing larger sections of lip to allow access of reagents into the deeper tissue. To overcome this problem the protocol can easily be combined with a de-epithelializing first step, such as hypertonic saline as demonstrated in protocol C. An alternative to using hypertonic saline would be to use trypsin as utilised in protocol D. However as discussed in the introduction (section 1.2.2) trypsin causes disruption to ECM proteins especially laminin, fibronectin and elastin. This is evident in the current experiment, where both protocols which used trypsin (protocol A and C) resulted in almost complete loss of elastic and destruction on the ECM microstructure as a result.

Now that a successful protocol has been identified for the decellularization of human lip it must be refined and tested. Further studies should involve quantitative analysis of the effect of this protocol on ECM components and formal testing of its mechanical properties. The microstructure of the scaffold should be further characterised with either scanning electron microscopy (SEM) or transmission electron microscopy (TEM) to get a detailed 3-dimentional image of the ECM. Once scaffold preparation has been optimised in-vitro cytocompatibility testing should be carried out, to assess the ability of the scaffold to support cell culture.

Conclusion

The present study shows for the first time that the human lip can be decellularized as a complete construct, preserving the natural structure of the lip and the unique composition of the ECM. The study has identified that a non-detergent, non-trypsin based protocol, combined with a de-epithelializing first step provides the optimal method for removing all cellular matter form the tissue, whilst also preserving the major components of the ECM. The acellular scaffold produced may be the first step towards an exciting new treatment for perioral tissue loss. Further experiments are now required to assess the mechanical properties, 3D architecture and cytocompatibility of this scaffold.

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