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Histological and ultrastructural characterisation as minimal criteria for assessing the success of the decellularisation protocols for tissue engineering applications#

K. G. Megha[1], Dhanush Krishna Balakrishnan Nair[2*], C. Divya[2], I. S. Sajitha[2], V. N. Vasudevan [3], P. R. Umashankar[4] and S.S. Devi[2]
Department of Veterinary Pathology,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University, Kerala, India

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Abstract

There is a great demand of suitable biomaterials for tissue engineering applications in regenerative medicine. Decellularized extracellular matrix scaffolds derived from different organs and tissues have been successfully applied for human use as therapeutic agents in many tissue defects. The aim of the present study was to establish histological and ultrastructural characterisation as minimal criteria for assessing the suitability of decellularised bioscaffolds in tissue engineering applications. The study was performed on bovine omentum processed by a natural bovine bile based decellularisation protocol. Histological analysis and scanning electron microscopy examinations were performed and compared with an established decellularised bovine pericardial scaffold. Gross examination revealed that the decellularisation process did not alter the shape of the omentum. Histological examination confirmed the preservation of the collagenous fibres without any observable cell nuclei following decellularisation. Further, scanning electron microscopy examination demonstrated the arrangement and ultrastructure of collagen fibrils in the bioscaffolds. Our results suggest that histological and ultrastructural characterisation provide preliminary data on determining the biocompatibility of scaffolds before planning in vivo studies in tissue engineering applications.

Keywords: *Biomaterial, bovine omentum, decellularisation*

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1. *MVSc Scholar*
2. *Assistant Professor*
3. *Assistant Professor, Department of Livestock Products Technology*
4. *Scientist 'G' & Scientist-in-Charge, Division of In Vivo Models and Testing, Biomedical Technology Wing, Sree Chitra Thirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala-695 012*

** Corresponding author: dhanush@kvasu.ac.in*

Organ transplantation is one of the great advances in modern medicine. However, the need for organ donors is much higher than the number of people who actually donate (Panwar *et al*., 2016). Hence, research in regenerative medicine seeks alternative approaches for treatment in tissue and organ failure using extracellular matrix (ECM) (Balakrishnan-Nair *et al*., 2019). Decellularisation is a promising technique in tissue engineering where the ECM is isolated from its native cells and genetic material in order to produce a natural scaffold (Balakrishnan-Nair *et al*., 2018). An ideal decellularisation process maintains structural, biochemical and biomechanical characteristics of ECM scaffolds, which can then be recellularised to produce a functional tissue or organ. Decellularisation can be achieved using natural, chemical, enzymatic, physical or combinative methods, where each strategy has its own merits and demerits (Xing *et al*., 2015). The decellularisation process can also alter the composition of ECM which can impact the outcome of various biomedical applications (Lin *et al*., 2019).

A properly decellularised ECM based bioscaffold preserves the amount of collagen, pore size, glycosaminoglycans (GAGs) and growth factors (Allen *et al*., 2010). Thus, these scaffolds improve the success rates in various clinical applications. However, poorly decellularised ECM bioscaffolds produce adverse immune reactions leading to rejection of the materials (Aamodt and Grainger, 2016). There is a need for establishing minimal criteria for assessing the success of decellularisation protocols. Hence, the present study proposes minimal criteria for assessing the success of the decellularised bioscaffolds based on histological, histochemical and ultrastructural studies for tissue engineering applications in repairing bioscaffolds. The ultimate aim of tissue engineering is to develop low cost and effective next generation "off the shelf" ECM based solution for clinical applications.

Materials and methods

Preparation of scaffolds

Fresh bovine omenta were collected from Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy at the time of slaughter. Manual delamination was done to separate omental layers. Defattening was achieved by manual and chemical (chloroform: methanol; 2:1) methods. Defattened omental scaffolds were subjected to decellularisation process with 80 per cent aqueous bile in a shaker incubator followed by washing with 1 per cent Tris Buffered Saline with Tween-20 (TBST) and later dried under laminar air flow (Ashna *et al*., 2019). The sheets were well packaged in a polythene bag and sterilised by gamma irradiation at 25KGy (BRIT, Mumbai, India) (Ashna *et al*., 2019). Decellularised bovine pericardium procured from Division of *In Vivo* Models and Testing, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, was used as control.

Microscopic evaluation

Representative samples of decellularised bovine omentum and decellularised bovine pericardium were fixed in 10 per cent neutral buffered formalin solution (10% NBF). These scaffolds were then washed and dehydrated, embedded in paraffin wax and sectioned at 4-5µm thickness. The sections were stained with haematoxylin and eosin (H&E), Masson's trichrome (connective tissue stain) and alcian blue stains (glycosaminoglycans) (Suvarna *et al*., 2018).

Scanning electron microscopy (SEM)

The representative samples of decellularised bovine omentum and decellularised bovine pericardium were fixed in 2.5 per cent glutaraldehyde solution for 48 h. The samples were then washed thrice with buffer (0.1 M PBS) for 10 min each. The samples were then dehydrated through ascending grades of alcohol (50%, 60%, 70%, 80%, 90% and 100 %) for 45 min each. The samples were air dried and mounted on sample stub having double sided carbon adhesive. Later, the samples were sputter coated with a thin layer of gold and analysed using scanning electron microscope (Hitachi 3000N) at an accelerated voltage of 15kV and micrographs were taken.

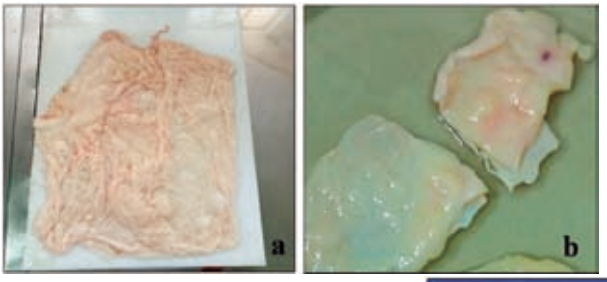


Fig. 1.
a. Fresh bovine omentum
b. Fresh bovine pericardium

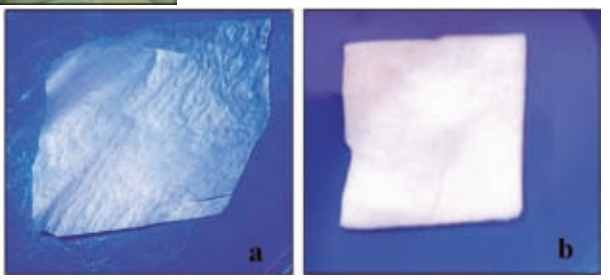


Fig. 2.
a. Decellularised bovine omentum
b. Decellularised bovine pericardium

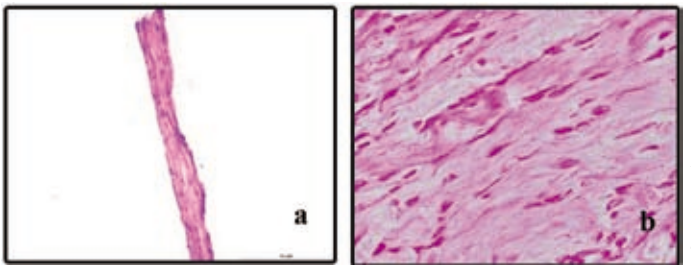
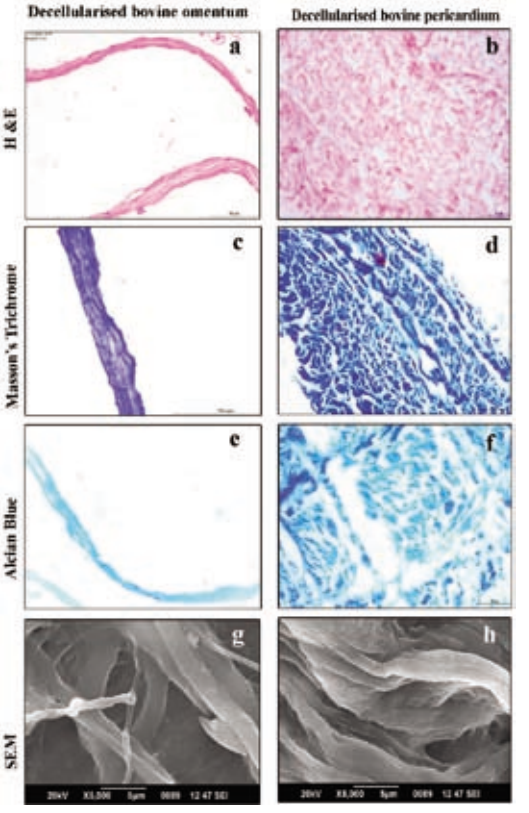


Fig. 3. a. Non decellularised bovine omentum showing nucleus in the matrix (H&E X 400) b. Non decellularised bovine pericardium showing nucleus in the matrix (H&E X 400)

Fig. 4.

a. Decellularised bovine omental scaffold showed pink coloured cytoplasm with absence of nuclear remnants (H&E X 400) b. Decellularised bovine pericardial scaffold showed pink coloured cytoplasm with absence of nuclear remnants c. Decellularised bovine omental scaffold showed parallel collagen pattern with absence of nuclear remnants (Masson's Trichrome X 400), d. Decellularised bovine pericardiual scaffold showed parallel collagen pattern with absence of nuclear remnants (Masson's Trichrome X 400),e. Decellularised bovine omental scaffold showed presence of glycosaminoglycans in the decellularised matrix (Alcian blue stain X 400), f. Decellularised bovine pericardial scaffold showing presence of glycosaminoglycans in the decellularised matrix (Alcian blue stain X 400), g. Scanning electron micrograph of decellularised bovine omentum revealed acellular, intact and thick collagen fibers and pores were visible (red arrow)(SEM X 5000) h. Scanning electron micrograph of decellularised bovine pericardium revealed acellular collagen fibers and pores (red arrow) (SEM X 5000)



Results and discussion

Decellularised omenta were prepared from native tissues (Fig. 1) using a natural bile based decellularisation protocol (Fig. 2). Hematoxylin and eosin (H& E) staining was performed on decellularised bovine omentum and decellularised bovine pericardium (Fig. 3). No nuclei or cell fragments were visible in both decellularised scaffolds up on H&E staining. In Masson's trichrome staining, blue coloured collagen was observed in decellularised bovine omental and pericardial scaffolds with the absence of black coloured nuclei (Fig. 2). Presence of glycosaminoglycans (GAGs) was demonstrated with alcian blue staining in decellularised bovine omental and pericardial scaffolds. In both decellularised bovine omental and pericardial scaffolds, the presence of GAGs was indicated in light blue color (Fig. 4). Scanning electron microscopy demonstrated collagen pattern and pores in between the collagen fibers of various scaffolds (Fig. 4). The decellularised bovine omental scaffolds and decellularised bovine pericardial scaffolds showed acellular wavy collagen pattern with sufficient pores in between collagen fibres.

Decellularisation is a critical point in tissue engineering which affect the architecture and composition of biomaterials. Different decellularisation protocols have been established to yield several tissue derived scaffolds for tissue engineering applications. According to Vasudevan *et al*. (2016), the bile decellularisation method was proven to be successful for removing most of the cellular contents from the matrix of omentum. Bovine bile is an abattoir waste that can be used as good decellularising agent for ECM matrices because of its detergent property attributed to the contents such as sodium cholate and sodium deoxycholate (Azum *et al*., 2019). This is *via* altering lipid membrane structure of cells leading to dispersion of lipid contents of cell membrane resulting in destruction of cells and effective removal of cells from the ECM matrix leaving scaffold network. After decellularisation, an ideal ECM scaffold is also expected to maintain the structure of collagen and elastin fibers as well as content of biochemical molecules such as GAGs. It has been noted that any disturbance caused in the architecture of collagen fibres in ECM can affect biomechanical properties of the scaffolds (Sheridan *et al*., 2012).

Functional and structural disturbances of ECM were not only associated with collagen fibres but also related to elastin degradation, glycosaminoglycans loss etc (Chen *et al*., 2004). Physical (freeze-thawing cycles, mechanical forces), chemical (ionic and non-ionic detergents; hypertonic or hypotonic salt solutions, and acids and bases) and biological methods (enzymes) have been extensively used for decellularisation (Dussoyer *et al*., 2020). In addition, nucleases are used to promote the fragmentation of residual DNA into <200 bp fragments in order to minimise immunological responses (Fernandez-Perez and Ahearne, 2019). Decellularisation was said to be effective when DNA fragment length was between 100- 200 base pair and also DNA dry weight in the decellularised matrix was less than 50ng. Extensive research has been conducted to optimize these decellularisation procedures to allow for maximal cell removal and minimal ECM damage for each tissue/ organ. The present study was to establish histological and ultrastructural characterisation as minimal criteria for assessing the success of the decellularised bioscaffolds following various Decellularisation protocols for tissue engineering applications. Decellularisation can remove cellular components; otherwise, which can induce residual immunological response in host (Wong and Griffiths, 2014). Bile is an emerging decellularising agent owing to its chemical composition having decellularisation property. The efficiency of decellularisation process was studied by considering the absence of cell nuclei with H&E (He, 2020). Here, both scaffolds upon H&E staining showed absence of cellularity confirmed the effectiveness of decellularisation (Fig.2). Masson's trichrome staining revealed preservation of the collagenous fibres (Fig. 2), as well as porosity. Alcian blue staining was used to ascertain the presence GAGs in decellularised matrix (Fig. 2). Retention of naturally occurring GAGs in the decellularised matrix can act as a factor for cell growth due to its ability to bind growth factors and cytokines

and also owing to its water retention property (Xing *et al*., 2014). Glycosaminoglycans have important role in growth, migration and proliferation of cells (Salbach *et al*., 2012).

In the present study, scanning electron microscopy images revealed microarchitecture of acellular collagen fibres with sufficient pore sizes in both decellularised bovine omental and pericardial scaffolds which is beneficial for recellularisation and cell growth (Fig. 2). Both decellularised bovine omental and pericardial scaffolds showed acellular, intact and thick collagen fibres, with sufficient pores size. According to the observations made by Porzionato *et al*. (2013) decellularisation of omentum resulted in removal of lipid and cellular components from the matrix but preserved the architecture of collagen, elastin and reticular fibres and glycosaminoglycans needed for regeneration applications. We therefore suggest histological and ultrastructural characterisation of ECM bioscaffolds as the most basic and minimal criteria for assessing the efficiency of decellularisation protocols. Advanced immunological assessment of scaffolds by means of special nuclear stains like DAPI, Hoechst 33342, Hoechst 33258 etc and DNA quantification employing nanodrop spectrophotometry can be further employed for confirmation.

Conclusion

In future studies, we can develop cell quantification approach as a strategy using H&E stained sections for tissue engineering applications. In the present study, histological and ultrastructural characterisation provide preliminary data on determining the biocompatibility of scaffolds before planning biomechanical testing or *in vivo* studies for tissue engineering applications. Hence, histological and ultrastructural characterisation can be considered as minimal criteria for assessing the success of the decellularisation protocols for tissue engineering applications.

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Conflicts of interest

The authors declare that there is no conflicts of interest**.**

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