

CELL-SEEDED DECELLULARISED EXTRACELLULAR MATRICES AS AN ADVANCED APPROACH FOR TISSUE ENGINEERING

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Abstract.

Development and optimisation of therapeutic applications using biological scaffolds composed of naturally occurring extracellular matrix (ECM) have received significant attention in the field of tissue engineering. Histological and molecular investigations combined with comprehensive electron microscopy have shown that decellularised matrices from animal sources may vary in structure and biochemical properties. The source (bladder or small intestine submucosa), species, age of animal, and type of preparation of these matrices may contribute to the unique biophysical properties of the delivered product. Comparison of the thickness, topography, porosity and biomechanical properties of these matrices are presented. Vascular cell adhesion, migration and proliferation, and cell ingrowths on scaffold matrices were analysed using fluorescent labelling and laser confocal microscopy. ECM is shown to be a promising material for vascular tissue engineering and supports the growth, proliferation, anchoring and migration of human vasculature derived cells. Scaffold features tightly control the rate of adhesion and initiation of the proliferation. UBM scaffold containing an intact basal membrane may strongly influence the phenotype of the smooth muscle cells and control their proliferation-differentiation mechanisms. Data obtained will help realise and guide the research community with regard to the appropriate use of extracellular matrices derived from various animal organs based on their properties and suggests the possibility of conditioning this matrixes in order to influence their tissue and cell specificity before utilisation in clinical use.

INTRODUCTION

Tissue-engineering experiences over the last decade are shown that one of the keys component of the development of the functionally is the choice of 3-D scaffold with which tissue growth is guided [1]. Major characteristics of the scaffold not only accommodate and guide cell growth in the three-dimensional space. A structural features such as the orientation of these fibers, affinity of the chemical groups on the surface of the scaffold have to support the attachment of the cells by the certain type of the integrins which will stimulate a correct proliferation rate of the cell, allow healthy mechano-transduction pathway mechanisms under the physiological flow and do not trigger production of a pro-inflammatory signaling molecules [2,3,4]. The list of 3D -scaffold materials continues to grow with development of new polymeric compounds and fabrication techniques [2]. Naturally derived materials offer many mechanical, chemical and biological advantages over synthetic materials, and thus hold tremendous potential for use in tissue engineering therapies. Tissue-engineering approaches utilizing the

decellularisation of the animal organs in order to obtain an advanced 3D- scaffold (decellularised Extracellular matrices –ECM) may serve as potential alternatives for synthetic polymeric materials in vascular medicine. This techniques are has typically required chemical or physical pretreatment aimed to preserve the tissue, enhance the resistance of the material to enzymatic degradation, and reduce the immunogenicity of the material. The distribution of natural polymers, their ratio, cross-linking pattern, elasticity, presence of the growth factors, total chemical composition and structure of the extracellular matrix are influenced by biomechanics and physiological function of the different source organ, and allow organ to function in an efficient manner and perform its unique organ function [6]. Limited number studies conducted biochemicals tests have been analyze the composition of decellularised matrices. Several authors applied histological tools to show 2 D- distribution of the structural component within the matrices, but are mostly limited by elastin and collagen content which are known to be the major content of the ECM matrix [7,8]. A lack of information and understanding of the spatial and dimensional distribution of different matrix components such as porosity and fibres thickness within the variety of the matrices led us to focus the current research on the detailed characterization the topographical features and structure of the polymer in 3D- distribution of natural component in the of the intracellular matrices derived from sub-intestinal submucosa (SIS) [6, 7] and urinary bladder matrix (UBM) [8]. These matrices have been successfully commercially applied in the regenerative medicine of the epidermis and skin. But in order to apply this scaffolds application of this in the challenging cardiovascular area, where potential failure of the graft may lead to sever problems even death, more data needs to be obtained to characterize this type of the material: their potential, their limitation and potential biological effect on the cells, which may be in contact with the scaffold. Recently, a few *in vivo* studies demonstrated an inconsistent performance of this material and the publications did not containing sufficient data on the complications. Source of the possible complication may only be hypothesized as follows:(a) diameter and anatomy of the vessels where material are introduced have been shown to have a defining role in the rate of the successful integration of the graft into the host tissue;(b) blood –compatibility and thrombogenicity of this material are not evaluated and understood;(c) certain ratio of the early calcification of the decellularised tissue need to be investigated; (d) the performance of the various decellularised matrices correlate to multiplicity of the application and tissue-specific response in different organ replacement therapies. A possibility that the origin of the ECM, achievable biomechanical properties, structural components, variability between samples derived from animal to animal, age and gender, deposition of the structural component and growth factor are important and influencing factors and reflects on outcome of their clinical performance. The data or comparative assessment of the different material was not verified by other workers. The objective of this study was to inspect the mechanical and biological characteristics and provide an comparative data of the main features of two decellularised matrices extracted from small intestine submucosa (SIS) and Urinary Bladder Matrix (UBM).

2. MATERIAL AND METHODS

2.1 Origin of extracellular matrices

The preparations of Urinary Bladder Matrix (UBM) and Small Intestinal Submucosa (SIS) have been previously described [6,7,8].

2.2. Scanning electron microscopy

The surface morphology of the scaffolds and cell morphology post *in vitro* cell seeding study was examined using scanning electron microscopy (SEM). The specimens were washed with PBS three times, than fixed in 2.5% glutaraldehyde at 4°C and rinsed in PBS. All samples were post-fixed with 1% OsO₄ for 1 h followed by a dehydration process through a graded series of ethanol (30–100%, 10 min) and air dried in a fume hood. Gold spluttered samples were examined using a scanning electron microscope (SEM) at 5kV.

2.3 GAG content determination - Alcian Blue staining

The amount of glycosaminoglycans (GAGs) in the decellularised matrices was determined using the Alcian Blue staining assay [9]. The presence and distribution of GAG groups was qualitatively verified by the accumulation of blue stain using an optical microscope.

2.4. Biomechanical properties of the scaffolds

The ECM scaffolds of interest were cut into dog-bone shape specimens using a stainless steel template with dimensions as previously recommended [8]. Fibre direction and alignment were determined by a laser scanning confocal microscope (LSCM) system equipped with spectral detection system Meta 710 (Carl Zeiss). Figure 1 illustrates the sample preparation and test set-up.

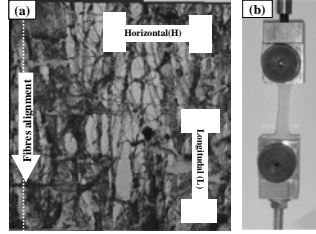


Fig.1 (a) Schematic representation of the sample preparation from decellularised material for biomechanical testing and (b) *in situ* experimental apparatus

Samples were cut in the horizontal and longitudinal direction based on the fibre alignment determined for each sample analysed in this study. Specimens were immersed in PBS and allowed to hydrate for a short controlled period of time. The specimens were mounted in a uniaxial mechanical tensile testing machine developed in house; between two coarse screw-tightened grips to prevent slippage and maintaining an initial gauge length of 43mm. Specimens were loaded at a constant rate of 40mm/min until failure. A personal computer was connected to the testing machine and data recorded at a rate of 10 data points per second. Force and elongation values were recorded for each specimen. The maximum load (F_{max}), elongation-at-failure (e_{max}) and maximum tangential stiffness (K^i_{max}) were also obtained. The initial length (e_0) was measured at a load of 0.01N. The tangential stiffness was calculated as follows:

$$K^i = \frac{F^{i+10} - F^{i-10}}{e^{i+10} - e^{i-10}} \quad (1)$$

Where i is a data index indicator, F is the recorded force, and e is the recorded elongation. The maximum tangential stiffness was taken as the maximum value of K^i .

2.5. Repopulation of decellularised matrices by vascular cells *in vitro*

2.5.1. Cell lines and growth media

Human aortic smooth muscle cells (SMCs) and endothelial cells (ECs) (PromoCell, Germany) originated for a 25 year old male donor with no cardiovascular disease. Cell lines were maintained in SMC medium (PromoCell, Germany) (containing 10% FBS, 1% SMCGS supplement and 1% pen/strep solution, and EC growth medium-2 (EGM-2, Lonza) both supplemented with 10% FBS. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO₂.

2.5.2. Adhesion, migration, spread and ingrowth of vascular cells into the scaffold material

SMCs and ECs were seeded onto the luminal and abluminal sides of the lyophilized UBM and SIS 2.5 cm² disks at a density of 2×10^5 cells/cm² and 3×10^3 cells/cm² respectively. After 6 hours in culture, non-adherent cells were aspirated off. The viability of the SMCs and ECs on the scaffold matrix were

examined by Live/Dead assay (Invitrogen) at various time intervals. Briefly, cell seeded scaffolds were washed with PBS, incubated in calcein-AM (staining live cells) and EthD-1 (staining dead cells) in PBS for 30 min at RT. The localisation of cells on the surface and degree of penetration of seeded cells into the scaffold were visualised using LSCM.

2.6 Assessment of the maintenance of the of vascular cell phenotypes.

The total RNA was extracted and reverse-transcribed to produce cDNA. PCR reactions were performed with specific primers for *vWF* and *PECAM-1*, *SM- α actin*, *Smoothelin SM-Myosin Heavy Chain (SM-MHC)* [9]. Amplification products were separated with 1.5% agarose gel electrophoresis and visualised with EtBr. Sufficient elimination of the DNA from the sample was confirmed by PCR amplification of the RNA sample without reverse transcriptase reaction.

3. RESULTS AND DISCUSSION

3.1 Structural properties of the decellularised matrices used

Structural properties of the two ECM materials vary dramatically. UBM thickness ranged from 80-300 nm and was 7-10 folds thicker than the SIS scaffold. Thickness of the SIS material was in the range of 15-80 μ m. Representative images of material cross-sections are presented in Figure 2. UBM showed the capacity to be easily compressed.

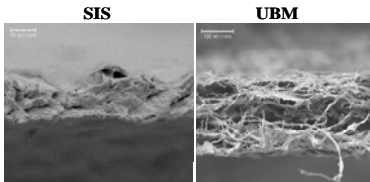


Fig.2 SEM micrograph of the cross-sections of the decellularised scaffolds

Analysis of the topographical material features showed that UBM and SIS scaffolds have bimodal surface characteristics. This was more pronounced in UBM as shown in Figure 3. Morphology of the luminal and abluminal surfaces has distinctive features and different degrees of roughness, fibre content and alignment and possibly cell spread and accommodation capabilities. Decellularised UBM contains a Basal Membrane (BM) which is absent in the SIS. BM is shown to be an important structural constituent of a blood vessel. Modulation of its structure is responsible for maintaining cell phenotype, proliferation rate of the ECs and SMCs and pro-inflammatory response on the shear stress. This study showed that mechanical extraction of the muscular components of the bladder can cause damage to occur in multiple regions which are often distributed over the graft surface and may serve as a 'nucleation site' for thrombogenic response and platelet infiltration. The degree of porosity of the UBM material is shown to increase with distance from the luminal to the abluminal side (size 15-70 μ m).

3.2 GAG content

Many different genetically distinct core proteins combine with various GAG chains to form macromolecules expressing specific features and perform numerous functions. They influence the mechanical properties of tissues, regulate collagen matrix organisation, form an anti-thrombogenic barrier in the vascular lumen, participate in cell-cell or cell-ECM interactions and bind growth factors and viruses [11, 12]. Figure 4 illustrates the GAG content in the decellularised scaffold. The intensity of the colour corresponds to the amount of GAG. Degree of the sulfated GAG, in the graft materials needs to be determined, such as lipid retention from the blood and may associate with increase thrombogenic response shown to correlate with this component

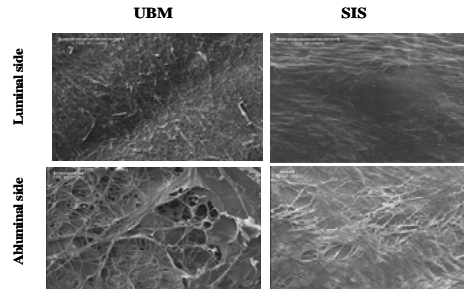


Fig.3. Composed gallery of the SEM micrographs obtained from luminal and abluminal surfaces of the decellularised scaffolds (UBM and SIS).

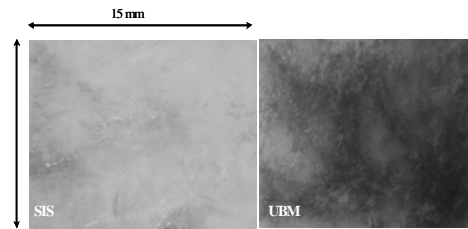


Fig.4. Variability in the GAG content of decellularised scaffolds (Alcian Blue staining).

3.3 Mechanical properties of the decellularised scaffolds

The uniaxial tensile test data of UBM and SIS is summarised in Figure 5. Architecture and alignment of scaffold fibre is shown to play a critical role in the mechanical properties of the ECM. This is shown by the higher strength and stiffness of SIS upon uniaxial tensile testing in the preferred longitudinal fibre direction when compared with a horizontal fibre alignment. A higher maximum force and stiffness was observed in SIS compared to UBM, however, UBM scaffold exhibited more elastic properties and higher elongation properties. This result may reflect the physiological mechanical functions of the organ from which the ECM is derived. UBM does not possess the mechanical strength of the tunica submucosa in SIS but has the ability to expand with applied stretch. The anisotropic characteristics of UBM and SIS needs to be elucidated and in conjunction with already obtained data, in order to formulate methods for the preparation of various scaffolds with different properties, to reconstruct a multilayered construct with desirable properties of porosity, structural constituents and excellent compliance.

3.4. Vascular cell phenotype maintenance, adherence and behaviour on the scaffold

This study assessed the influence of structural features on adhesion, proliferation and the growth dynamics of human aortic cells (ECs and SMCs). Cellular distribution and attachment to the matrix during *in vitro* static culture were determined. A 3D-reconstruction from 23 single images was obtained from various depths of the EC and SMC seeded scaffold. Visualisation and the pattern of cell repopulation were obtained at various times of the cell growth on the scaffold (Fig. 6 and 7).

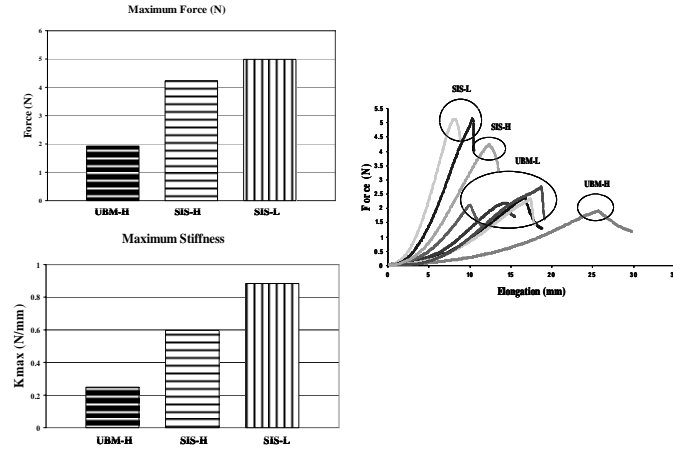


Fig.5. Biomechanical properties of UBM and SIS and their variability in break force, stiffness and elongation.

The spatially uniform distribution reflects porosity and surface charge of the scaffold. The luminal side of the UBM containing the BM shows excellent capacity to support the migration of ECs. However, the initiation of cell attachment was pronounced in the region that showed a high degree of porosity or exposure to a structural roughness.

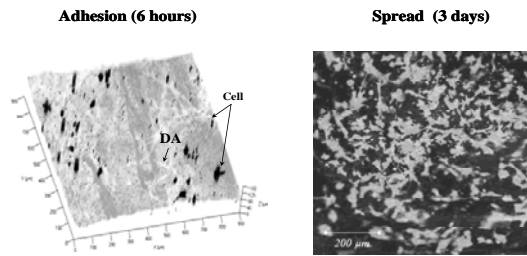


Fig.6. 3D-LSCM image of aortic ECs on luminal side of decellularised material (static condition); (a) – after 6 hours of adhesion, cell (shown in black) showed attachment to less dense packed scaffold (DA); (b) - the spread of ECs (shown in white) after 3 days of the growth on scaffold surface (grey)

The UBM material was shown to contain $150\mu\text{m}^2$ areas with a certain degree of BM damage. Monitoring of the fluorescence signal derived from live and metabolically active SMCs (Live/Dead Viability staining), the initial adhesion of the cells within these regions and pores is clearly visible and probably initiated by the higher degree of irregularity, roughness and availability of more chemical and structural groups for SMC attachment (Fig. 7) as similarly shown for the ECs (Fig. 6). To overcome this potential irregularity, additional aspects of cell-matrix cultivation should be considered. Dynamic stress stimuli may be beneficial for inducing evenly structured construct

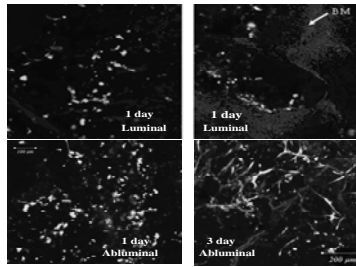


Fig.7. Adhesion and ingrowths of the SMC into the luminal and abluminal sides of the bimodal basement membrane containing decellularised material (UBM) (Cell (white), scaffold (grey), pore (black due to absence of fluorescent signal)).

The possible effect of the bioactive compounds from decellularised matrices (growth factors) on the phenotype of the seeded cell was evaluated. The expression of Von Willebrand factor (vWF) and PECAM-1 by ECs (7 days on the scaffold material) confirmed phenotype maintenance on UBM and SIS scaffold (Fig. 8).

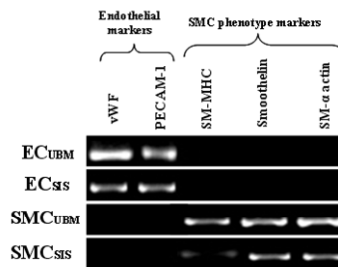


Fig.8. Results of the RT-PCR analysis of expression of phenotypic markers by the ECs and SMCs upon growth and proliferation of the decellularised matrices

SMCs shown to express the *smoothelin*, *SM-MHC* and *SM-α actin* on both constructs (Fig 8), with low level of the myosin transcript during the growth on the SIS matrix. This may support the suggestion that cells with prolonged incubation time may undergo the switch to synthetic phenotype and the time taken for this to occur is modulated by the properties of the scaffold utilised for the cell maintenance. The stimulation of the construct by various physiological and chemical factors (pulsative flow, EC metabolites, etc.) in order to maintain a balance between remodeling of the scaffold, synthesis of the ECM components and the growth of the new tissue are currently under investigation.

4. CONCLUSION

An enormous effort by multidisciplinary groups has been put into the development of tissue engineered blood vessel substitutes over the last number of years. Among the various approaches, decellularisation of the animal organ and tissues in order to produce a bioactive scaffold, has shown to be a promising application in other soft tissues and can be adopted for the generation of the cell-seeded scaffold to create a viable blood vessel analogue or provide a sufficient cell-deliveries system into the pathological vessel. This study has shown that decellularised matrices from the animal sources may vary greatly by its structural and biochemical properties. Animal species, age of the donor organ and type of preparation of these matrices may contribute to the unique biophysical properties of the delivered product. The biomechanical properties of the scaffolds are shown to be influenced and probably correlate to the

biomechanics of the original organ from which the ECM material had been extracted. In relation to vascular tissue engineering, ECM is shown to support growth, proliferation, anchoring and migration of EC and SMC cell types obtained from human vasculature. Scaffold features tightly control the rate and initiation of adhesion, proliferation and migration. An intact basal membrane in the UBM strongly influences the phenotype of the smooth muscle cell and controls their proliferation-differentiation switch. Data obtained in this study will highlight the presence of these differences between various ECM materials and guide the research community to the appropriate use of available or newly produced extracellular matrices derived from various animal organs. Based on the data presented the ability of these materials to support attachment and growth of human vascular cells and sufficiently maintain their phenotype, the use of in vitro bioreactors for the conditioning of these matrices in order to influence their tissue and cell specificity [13] and remove traces of animal pro-immunogenic constituents by remodeling with human vascular cells before utilisation in clinical use all need to be considered for further study.

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