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Assessment of Endothelial Cell Proliferation and Adhesion Strength on Decellularized Bovine Pericardial Scaffolds for Tissue Engineering

Pagoulatou Eirini, Mavrilas Dimosthenis*

Department of Mechanical Engineering and Aeronautics, University of Patras, Greece

***Corresponding author:** Mavrilas Dimosthenis, Laboratory of Biomechanics and Biomedical Engineering, Department of Mechanical Engineering and Aeronautics, University of Patras, 26500 Patras, Greece. Tel: +302610969488; Fax: +302610969464; Email: dmauril@upatras.gr

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Abstract

Decellularized animal tissues are attractive sources of scaffolds for tissue engineering applications. Their natural extracellular matrix is an interesting substrate for cell emigration, infiltration and attachment *in vivo*, enhancing cell function for the formation of new natural living tissue. In this work we investigated the role of the decellularization protocols in successful cell growth and attachment strength on the surface of decell animal tissues. For this purpose, we cultured bovine aortic endothelial cells on previously decellularized bovine pericardial tissues according to three different protocols. Microscopic analysis and application of shear stress on the scaffold surface was used for the assessment of cell growth and attachment strength. The results showed that the combination of mechanical force with detergent decellularization was superior compared with the enzymatic decellularization regarding cell proliferation on the scaffolds' surface. Cell attachment strength was satisfactory, even in high physiological stress levels. In conclusion, decellularized animal tissue can be considered as suitable scaffolds for tissue engineering.

Keywords: Cell Proliferation; Cell ECM Attachment; Decellularized Scaffolds

Introduction

The design of an ideal scaffold for Tissue Engineering (TE) has met the challenge of mimicking the extracellular matrix in composition and Micro-Nano topography, providing an increased speed in functional tissue regeneration or recovery of damaged tissues through supportive cell adhesion under appropriate guidance and cell signaling *in vivo*.

Cells within living tissues are surrounded by Extra Cellular Matrix (ECM) that supports cell adhesion via integrin receptors. In TE scaffolds, the ability of seeded cells to adhere to scaffold material is of paramount importance for potential regenerative cell response to biomechanical stimulation.

Cell-implant adhesive strength is a focused point in tissue engineering. Cells in physiological body circulation are subjected to varied mechanical stress fields including gravitational force, mechanical stretch or strain and shear stress. Due to the pulsatile nature of blood flow, blood vessels are subjected to significant variations in mechanical forces. The main challenge in TE scaffolds for vascular repair is the presence, integrity and state of endothelium lining at the implant - host interface [1-5]. Endothelial cells attached on the blood contacting scaffold surface (like heart valve leaflets or the lumen of blood vessel walls) function as an interface between blood and scaffold material. They play a crucial role as barriers for blood component interactions with scaffold and also detecting and responding to the mechanical forces generated by shear stress due to blood flow and scaffold's mechanical resistance. Shear stress can modulate endothelial cell functions by sequentially activating specific transcription factors, and the expression of genes and proteins [6,7]. Normal wall shear stress (1.5-2.5 Pa) promotes a quiescent endothelial cell state, suppressing proliferation, inflammation and apoptosis while promoting a protective anti-thrombotic and selective permeability barrier

[1,4]. The effects of externally applied shear forces have been used to determine the cell adhesion strength for cells attached to Extra Cellular Matrix (ECM) surface [7-9]. ln addition, activation of endothelial cells on biomaterials leads to expression of new adhesion molecules on their surface, controls the transfer of molecules and interacts with underlying cells to regulate their growth potential and proliferation [5,10]. Oppositely, dysfunctional endothelial cells promote inflammatory reactions, resulting therefore to scaffold/biomaterial rejection [10,11].

Cell adhesion and proliferation depend on the formation of the fibrous components and functional complexes of ECM [12-18]. Collagen receptors and collagen binding molecules [19], elastin peptides [20] and Glycosaminoglycans (GAGs) [21-25] seem to be responsible for chemotaxis, for organizing the ECM and for cellular communication. Treatment of allogenic biomaterials for decellularization to be used as implants in humans may highly preserve the structural integrity of many ECM proteins and is thus currently utilized for soft tissue repair applications.

Among the different strategies in the field of scaffold design for cardiovascular tissue engineering, our basic approach is focused to the use of decellularized allogeneic materials with structural similarity to the native cardiovascular tissues. Decellularized bovine pericardium was selected as a candidate for producing cardiovascular scaffolds, due to its successful behavior under dynamic mechanical loading and blood interaction *in vivo* after a long time use as bioprosthetic biomaterial. Biomechanical performance, structural integrity and composition of ECM, especially Glycosaminoglycans (GAGs) content, seemed to be preserved after decellularization using detergent treatment, as alternative to enzymatic decellularization. Endothelial cell survival and proliferation were also enhanced, as proved by *in vitro* cell culture studies [26].

Cell attachment on biomaterial surfaces has been extensively studied by measuring the detachment strength of cells from the surfaces, using two experimental methodologies: parallel plate flow chambers [27,28] or rotating disc/flow devices [29,30]. The spinning disc provided a more quantitative assay for the determination of cell adhesion, demonstrating a linear relationship between the force necessary to detach cells and the number of adhesive bonds [9,29].

The aim of this work was to explore cell - ECM interactions of Bovine Aortic Endothelial Cells (BAEC) attached on decellularized Bovine Pericardial (BP) tissues by quantifying the adhesion strength, as determined by exposing cells seeded on acellular BP surface to a shear stress field and measuring the cells remained attached. The adhesion and proliferation of cells on acellular BP were characterized by fluorescence and scanning electron microscopy. A spinning disc device was used to produce the shear forces field applied to the cells. Cell detachment was detected and compared with different commercially available acellular biomaterials.

Materials and Methods

A General Description of Bio Scaffolds

The method of decellularization for bovine pericardium has been described previously [26]. In brief, fresh BP obtained from the local slaughterhouse was decellularized by the detergent and enzyme extraction method. For the former method, the pericardium was incubated in hypotonic buffer (2D distilled water) for 2 hours at 4˚C and subsequently in hypertonic Tris buffer with 1% Triton® X–100 (AppliChem), 0.1% SDS (Merck), 150mM NaCl (Merck), 1% deoxycholic acid (AppliChem) and protease inhibitor (P1860 - Sigma Aldrich) at 4˚C for 12 hours. In enzymatic decellularization method, BP was agitated in Tris buffer with 20 μg/ml RNase and 0.2 mg/ml DNase (Applichem) in Trypsin/EDTA hypotonic Tris buffer solution (0.5 %/0.2 %, 10mM Tris, pH 7.5) for 48 hours at 37˚C. Finally, treated acellular BP under both modes was washed with PBS followed by cell culture. In addition to tissues treated using the above-mentioned protocols, commercially available acellular bovine pericardial patches for abdominal and vaginal wall (Synovis - Veritas Collagen Matrix) [31], kindly supplied by the company, were comparatively cultured.

Preparation of Cell Culture

For the study of the cell–material interactions, BAEC cell line BW-6001 (Lonza) was used. The cells were cultured in 25 cm^2 culture flasks in Dubelcco's Modified Eagle's Medium (DMEM - Biochrom) with 10% Fetal Bovine Serum (FBS - Biochrom) and 1% antibiotics (streptomycin and penicillin - Biochrom) at 37˚C with 5% CO_2 in a humidified incubator. The media were changed every two days. Cells were monitored daily using phase contrast microscopy, then sub cultured when they were confluent. The cultured endothelial cells were identified by FITC labeled FDA (Fluorescein Diacetate, 4μgr/ml working solution - Sigma Aldrich) using fluorescence microscopy (Nikon eclipse 80i with Nikon digital sight DS-L1). Cells grown to a 90% confluence were trypsin zed and transferred on the biomaterial's surfaces.

Cell Seeding Procedure

Acellular BP specimen from the three groups (Triton, Trypsin/EDTA and Synovis - Veritas) cut into 10 mm diameter discs were placed into separate wells of a 24-well plate. The fibrous layer of the matrix was facing down so that cells would be seeded on their initial mesothelial (heart-facing *in vivo*) surface. The samples were sterilized prior to endothelialization using UV lamb in the laminar flow chamber. Afterwards, they were incubated in supplemented DMEM medium overnight. Cultured cells were harvested from the culture flasks using 0,05% Trypsin/EDTA solutions (Bio chrome) and re suspended in the culture medium to a concentration of 1×10^4 cells/cm^2. Then the pellets of the re suspended cells were transferred and placed on the specimens' surface. After 30 min of incubation, the culture media was completed to a total volume 2ml per well. Seeded cells were cultured for a period up to six days,

during which the medium was changed every other day.

Characterization of Attached Endothelial Cells

Fluorescent Microscopy

After the seeding period, changes in the cell viability and morphology due to cell material interactions (attachment) were analyzed by indirect fluorescent staining and fluorescent microscopy. Cell viability was detected with the use of the live dye Fluorescein Diacetate (FDA), by which the viable cells were stained green. DAPI - phalloidin (Sigma Aldrich) double staining was used to identify blue stained cell nucleus and green actin filaments of endothelia attached on acellular bovine pericardial materials. Subsequent microscopic analyses were used to confirm homogeneity of surface cell distribution as well local surface cell density.

Scanning Electron Microscopy

The cell-seeded pericardia were fixed in 2.5% glutaraldehyde solution for 20 min, dehydrated with a graded series of ethanol, and dried at 4˚C overnight. The dried samples were sputter coated with gold and the pericardial samples were examined using a scanning electron microscope (SEM) (Zeiss Evo Ma10). The cell - cell and cell - pericardium interactions were thus observed and analyzed.

Adhesion Assays

Description of Spinning Disc Device

The details of the test apparatus have been described elsewhere [29]. In brief, it consists mainly of a cylindrical chamber made of Plexiglas® containing PBS buffer (pH 7.5, room temperature), and a stainless-steel shaft, ended to a circular disc to hold the sample discs, rotated by a DC electric motor (Figure 1). Each specimen disc was glued to the holder faced to the bottom of the chamber. The motor allowed the rotation of the disc in the buffer under controlled rotation speeds. Four triangular plates mounted perpendicularly at the bottom of the chamber and a collar around the rotating disc holder prevent or minimize the rotation of the bulk liquid in the plane of the disc and secure laminar fluid flow even at maximum rotational speeds (315 rads/sec). During rotation of the disc into the immobilized buffer solution a shear stress field was exerted on the endothelial cells, depending on the speed of rotation, which causes their partial detachment.

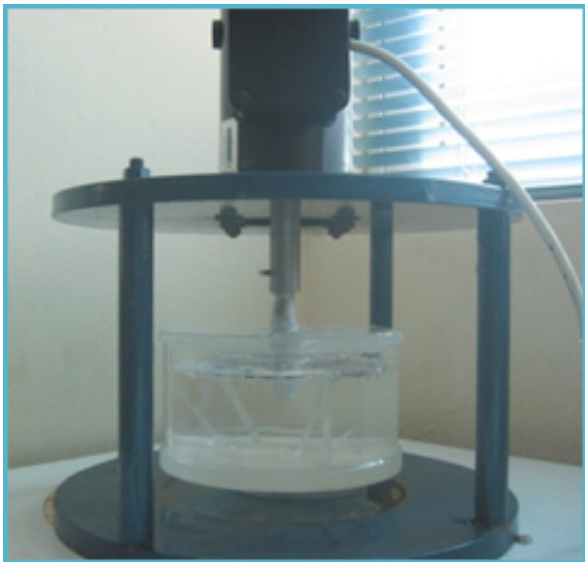

Figure 1: Photograph of the spinning disk device, used to produce a shear stress field induced on the endothelial cells cultured on acellular bovine pericardial biomaterial, by rotating the disks into an immobilized buffer solution.

An angular velocity $\omega = 230.2$ rad/s was applied to the motor resulting in a linearly increased shear stress field ranged from zero (center) to a maximum 124.37 dyn/cm^2 at edge (Figure 2).

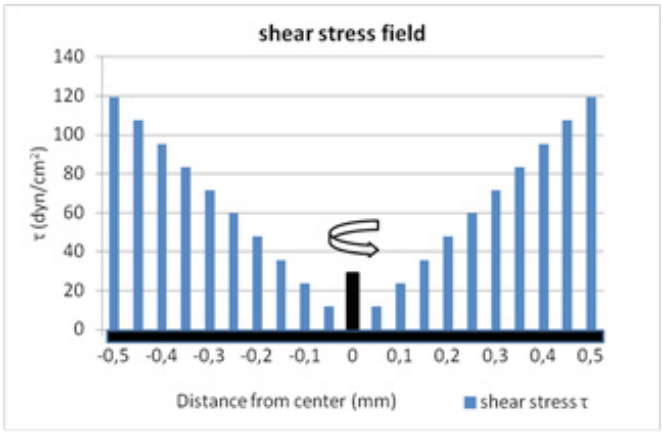

Figure 2: Diagram of the shear stress field developed across the disk diameter (zero at center to a maximum at the periphery of the disc).Cell Detachment Experiments

After a four days' incubation period in a 24-well plate the samples were spun in the spinning disc device for 10 min with a rotation speed 2199 rpm (230.2 rad/s). The hydrodynamic shear gradient applied to the cell population caused the detachment of cells if the shear stress exceeded the total strength of the bonds attaching the cell on the substrate. After the end of spinning testing the disks were removed, cells' nuclei stained (DAPI) and stepwise surface density of the cells remained attached, in 1mm^2 square frame steps, was measured across two rectangular specimen diameters by fluorescence microscopy and averaged. Detachment of the cells, as a percentage of local cell density divided by that of the central point (considered as 100% due to zero local stress) was thus determined with respect of τ.

Statistical Analysis

Data were expressed as mean values +/- standard deviation. Continuous data among groups were compared using repeated measurement analysis of variance followed by 95% confidence interval of the difference among studied materials (T-test). $p<0.05$ was considered as significant. Analysis was performed using SPSS for Windows, release 17.0.0 (SPSS Inc, Chicago, IL, USA).

Results

Cell Attachment and Proliferation on Scaffold

Endothelial cells cultured on decellularized BP scaffolds were viable and showed good proliferation as ascertained by fluorescence microscopy. Cell adhesion was monitored as soon as 2 hours after culturing the cells on acellular BP surfaces (Figure 3). Even at this short time a small cell population had already adhered to the pericardium, indicating that its surface is a suitable substrate for cell growth. Cell proliferation and growth was monitored over periods of 24 hours to 6 days of culture. On 6th day, the surface of the biomaterials had become coated 100% with cells. The mechanisms of cell attachment, including structural elements such as the cell cytoskeleton and focal adhesions were examined using immune fluorescent labeling of actin filaments (Phalloidin) and nuclei (DAPI).

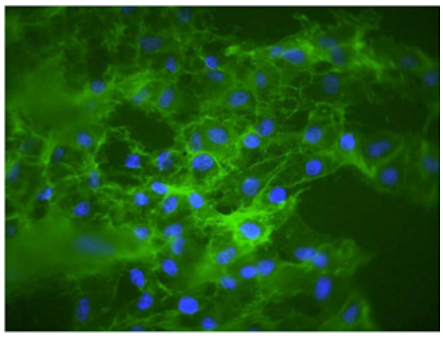

Figure 3: Characteristic image of fluorescence microscopy determining adhesion of bovine aortic endothelial cells on decellularized BP surface, after double staining with phalloidin - DAPI. Nuclei in blue and actin in green, magnification 20x.

Figure 4 shows endothelial cells lining after 4 days' cell culture, building a confluent monolayer on the inner surface of Triton (A) and Trypsin/EDTA (B) decellularized BP tissues. Similar cell configuration was detected on BP acellular matrix and on Synovis –Veritas material (C). Microscopic analysis confirmed the homogeneous distribution of cells on bovine pericardial tissue surface (approximately 95% confluence).The averaged local cell density at the center and at different step distances towards the perimeter of the disk samples of three groups was 151.6±19.2 cells/mm^2 for Triton BP, significantly greater than for Trypsin BP (99.0±17.1 cells/mm^2, p=0.048) and for Synovis - Veritas (91.2±31.9 cells/mm^2, p=0,047) (n=5, average±stdev).

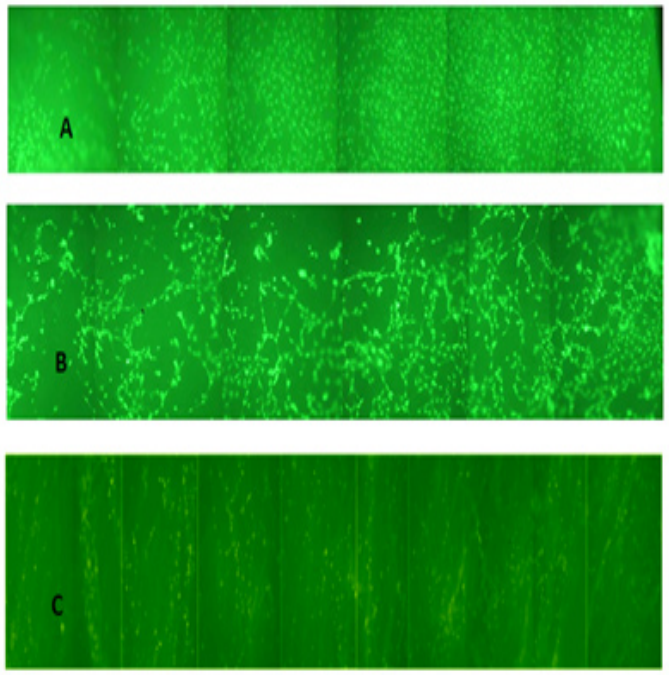

Figure 4: Representative images showing bovine aortic endothelial cell growth on the surface of acellular bovine pericardial tissue after 4 days of culture. A - Triton decellularization, B -Trypsin decellularization C - Synovis Veritas. Staining for live cells, FDA. Magnification 20x.

Cell Adhesion

SEM Analysis of Cell Morphology

Cell adhesion was determined by their pseudopodia developed towards BP surface, as demonstrated in SEM photomicrographs (Figure 5). It clearly shows the presence of BAEC developed pseudopodia attached on to BP surface. Intercellular interactions are also showed, as multiple cells are attached in a way to build tight cell junctions and prominent intercellular adhesion (A1 and A2).

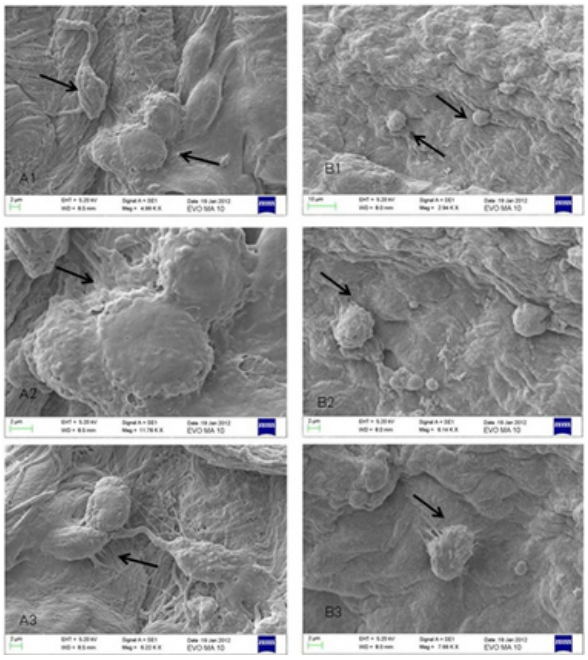

Figure 5: SEM photomicrographs of bovine aortic endothelial cells (BAEC) cultured on acellular pericardial tissues treated with Triton X-100/SDS (A 1-3) and Trypsin/EDTA solution (B 1-3), for 4 days. The presence of developed pseudopodia (arrows) attached on to BP surface is clearly indicated.

Analysis of Cell Adhesion Strength

As cells, cultured for four days, adhered in different positions across the diameter of the specimen disc, they were imposed in different shear stress levels during disk spinning, higher in longer distance from the center at a given rotational velocity (Figure 2). After spinning tests, cells that remained attached were microscope examined and photographed across two vertical diameters. Sequential microscope pictures of quadrant format 1mm^2 were then spliced and the local cell densities were measured using Sigma Scan Pro 5 software. Figure 6 shows a sequence of such pictures across two vertical diameters, demonstrating that the highest cell density measured at the center (τ=0), gradually reduced towards the perimeter, at highest stress (τ_{max}).

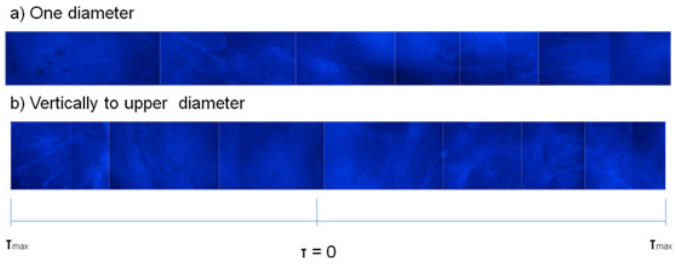

Figure 6: Sequential images of the surface of the disc with bovine aortic endothelial cells to form the diameter after rotation.

The results from the measured cell density averaged across vertical diameters showed that application of shear stress field τ gradually increased cell detachment from the center to the edges, following corresponding increment of τ (Table 1).

Shear stress,τ	τ= 0 dyn/cm^2	τ= 15 dyn/cm^2	τ= 40 dyn/cm^2
Materials	Cell density (cells/mm^2)		
Triton BP	145.5±8.7	103.3 ± 4.9	76.0±15.4
Trypsin BP	89.8±46.3	80.0±17.6	63.4±2
Synovis-Veritas BP	94.0±31.0	90.24±29.7	87.42±28.6

Table 1: Cell density (averaged across vertical diameters for each disk specimen) at different shear stress (Mean +/- SDEV, n=4).

Supposing no cell detachment at central region (where τ limits to zero), the absolute value of cell density after four days' culture for Triton BP was significantly greater than for Trypsin BP and for Synovis-Veritas (mean #cells/mm^2 +/- SDEV, n=4).

A reduced surface cell density, with respect to 100% at center, was computed to assess the attachment strength of the cells to the surface of the scaffolds, expressed as % cells remained attached after imposing in different shear strain levels. Diagrams in figure 7 show a gradual, near exponential, decrease in reduced cell density from 100% at central spinning disk region to a minimum 15.53 ± 5.21% for Triton decellularization, at disk edge region where maximum τ was applied. Trypsin/EDTA treatment exhibited a significantly higher reduced cell density (43.10 ± 10%) (p=0.001) at

edge, while the Veritas-Synovis acellular BP patches showed almost uniform distribution without significant cell detachment after spinning testing across the spinning disk diameter (mean ± SDEV, n=4 for all). The rectangular frame in the diagrams of Figure 7 focused in the physiological shear stress range (15 to 40 dyn/cm^2) like that applied at the lumen of blood vessel walls during normal blood circulation [32]. Within that range approximately 75-55% of the cells remained attached to Triton, 90-70% to Trypsin-EDTA and 96-93% to Synovis-Veritas acellular BP surface (corresponding cell density at Table 1). At the higher physiological shear stress (40dyn/cm^2), all the three scaffold materials demonstrated similar cell attachment strength (non-significant differences were detected).

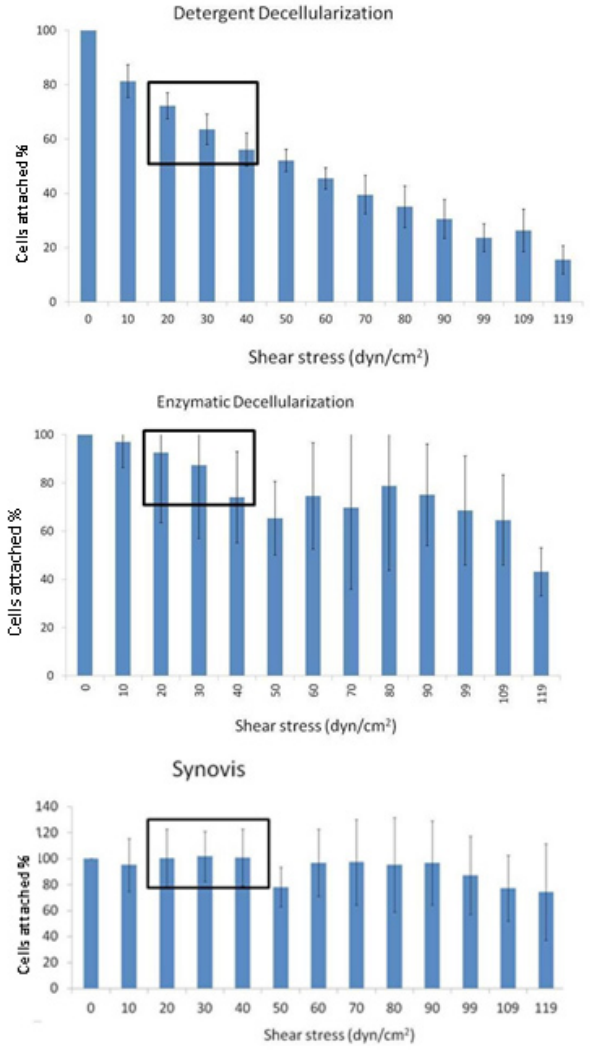

Figure 7: Experimental data of the count of adhered bovine aortic endothelial cells on BP substrates prior decellularized with Triton detergent and Trypsin/EDTA enzymatic protocols, together with Veritas-Synovis supplied acellular BP patches, after the application of shear stress field, using the rotating disc apparatus.

Discussion

For the creation of functional scaffold for tissue engineering that mimic the native soft tissue as closely as possible, *in vitro* studies of the interaction between cells and biomaterials need to be addressed. This cell-biomaterial interface is strongly related to the composition and structure of scaffold material and its ability to provide cell-specific protein junctions for growth of appropriate ligaments. The Extra-Cellular Matrix (ECM) of decellularized animal tissues may fulfill those requirements, provided that *in vivo* ECM structure and composition are preserved during decellularization. If so, acellular ECM may retain the proteins and appropriate biological indices, mechanical strength, resistance to enzymatic degradation and biocompatibility that have the potential to synthesize appropriate biochemical and biomechanical signaling to activate cell expression, differentiation and function towards tissue regeneration after implantation [33].

Previous studies on detergent and enzymatic treatment based decellularization of BP showed superiority of detergent method against enzymatic decellularization regarding ECM content and composition (especially for hyaluronan and other GAGs), as well mechanical behavior. In that work we successfully achieved full decellularization using detergents such as Triton, SDS, deoxycholate [26]. Several published works reported shortcomings of using SDS for decellularization such as difficulties in completely removing SDS molecules from tissues, cytotoxic effects and upregulation of elastases [34-36]. However, our preliminary results presented in the above mentioned work didn't show such effects.

In the present research we cultured BAEC on alternatively decellularized BP tissues and investigated the relationship of the resulted differences in ECM structure and mechanical properties, as demonstrated after decellularization, with endothelial cell attachment. After being cultured on scaffolds in static conditions, BAEC adhered and proliferated, typically forming tight cell structures on the scaffold surfaces (Figures 3 & 4). In that figures a uniform cell distribution on the surface of pericardial samples was showed with a good confluence at short time (2 hours) continued for longer time (95% and 100% confluence in 4 and six days). Again, no restrictions were presented due to the use of SDS for that periods (up to six days).

SEM micrographs (Figure 5) showed that binding between cell actin filaments and substrate surface was more evident in the case of Triton, compared with Trypsin/EDTA treated BP. This was quantitatively verified by the results of the shear stress application to the adhered cells by the spinning disc test. The spinning disc device was adopted to be used in combination with fluorescence microscopy, which allowed imaging, analyzing and counting of fluorescently stained cells on biomaterials before and after the application of appropriate shear stress field on their surfaces. This methodology enables an improved quantification of the adhesion strength since surface cell density before and after rotation at a given position can be accurately measured. Fine cell spreading and

proliferation on biomaterials' surface and good surface cell density was measured after 4 days' cultivation at the central disk region, where shear stress is minimal. The results in table 1 showed that absolute cell density demonstrated the superiority of Triton treatment against the Trypsin/EDTA, as measured at regions of minimal stress.

Cell density was decreased across the disk diameter with increasing the distance from the central point, hence increasing shear stress (Figures 2 and 6). The expression of cell counts by the reduced cell density presented here (Figure 7) is suitable for a direct objective comparison of the cell adhesion strength on the surface of different biomaterials under similar stress fields. The results showed a normal dependence of the reduced cell density to the shear strength for Triton-treated BP, an abnormal dependence for Trypsin/EDTA and practical no stress dependence for Trypsin based treatment for the Synovis-Verittas biomaterials. A direct comparison of absolute cell density, supposing uniform initial cell distribution over the surface of the disks seems to decrease the differences at maximum shear strength 120 dyn/cm^2, however even in that case the results showed a decreased resistant of the cells adhered on Triton treated BP, a medium resistance for Trypsin/EDTA and a great resistance for the Synovis-Verittas material.

This is evident that cells were adhered on the surface of ECM with different strength. This must have attributed to many reasons, like the surface chemistry of biomaterials, micro-Nano topography, cell population etc. The number of cells attached per surface area play an important role; if cell density increases, the possibility for the number of binding linkages of cell cytoskeleton directly to biomaterial surface is decreased, as some cells may adhere to other cells via intercellular binding linkages, as evident in Figures 5A1 & 5A2 [37,38]. It seems from the results that the higher cell density of Triton-treated BP contributed to the weaker cell attachment on the surface. Surface chemistry and micro-Nano topography were not studied, as it was beyond the scope of this work.

Looking however at the physiological stress range applied to endothelial cell during normal blood circulation (rectangular frames in the diagrams of Figure 7) it seems that such differences were minimized. Even at the higher physiological shear stress of 40 dyn/cm^2 a satisfactory percentage of cell remain attached to all the three biomaterial surfaces. Investigation towards the perfect scaffold for cardiovascular tissue engineering remains an enormous challenge. It is now unambiguous that cardiovascular scaffolds should fulfill several well-defined requisites: mechanical strength to withstand pressure in body, elasticity to provide compliance and recoil, cellular compatibility, ability to be repopulated and remodeled by host cells, lack of thrombogenicity and immunogenicity of the scaffold material and a confluent, shear resistant endothelium to resist thrombosis. An overall comparison regarding biochemical structure and content, biomechanical behavior [26] and capability for cell attachment between the biomaterials examined demonstrated the superiority of Triton against Trypsin based treatment for the creation of decellularized animal derived scaffolds.

In conclusion, successfully re-endothelialized acellular naturally derived biomaterial revealed cell-adhesion properties on biomaterial's surface, which are likely to be favorable to improve neo-tissue regenerative performance of biomaterials. Further research is in progress on undifferentiated mesenchymal stem cells seeded and cultured in bioreactor.

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