Physiological pulsatile flow culture conditions to generate functional endothelium on a sulfated silk fibroin nanofibrous scaffold

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Many studies have demonstrated that in vitro shear stress conditioning of endothelial cell-seeded small-diameter vascular grafts can improve cell retention and function. However, the laminar flow and pulsatile flow conditions which are commonly used in vascular tissue engineering and hemodynamic studies are quite different from the actual physiological pulsatile flow which is pulsatile in nature with typical pressure and flow waveforms. The actual physiological pulsatile flow leading to temporal and spatial variations of the wall shear stress may result in different phenotypes and functions of ECs. Thus, the aim of this study is to find out the best in vitro dynamic culture conditions to generate functional endothelium on sulfated silk fibroin nanofibrous scaffolds for small-diameter vascular tissue engineering. Rat aortic endothelial cells (RAECs) were seeded on sulfated silk fibroin nanofibrous scaffolds and cultured under three different patterns of flow conditioning, e.g., steady laminar flow (SLF), sinusoidal flow (SF), or physiological pulsatile flow (PPF) representative of a typical femoral distal pulse wave in vivo for up to 24 h. Cell morphology, cytoskeleton alignment, fibronectin assembly, apoptosis, and retention on the scaffolds were investigated and were compared between three different patterns of flow conditioning. The results showed that ECs responded differentially to different exposure time and different flow patterns. The actual PPF conditioning demonstrated excellent EC retention on sulfated silk fibroin scaffolds in comparison with SLF and SF, in addition to the alignment of cells in the direction of fluid flow, the formation of denser and regular F-actin microfilament bundles in the same direction, the assembly of thicker and highly crosslinked fibronectin, and the significant inhibition of cell apoptosis. Therefore, the actual PPF conditioning might contribute importantly to the generation of functional endothelium on a sulfated silk fibroin nanofibrous scaffold and thereby yield a thromboresistant luminal surface.

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1. Introduction

Since clinically available vascular prostheses do not offer satisfactory outcomes for the small-diameter (<6 mm) vessel replacements, the need for engineered small-diameter vascular grafts is real and urgent. A wide variety of natural proteins and synthetic scaffold materials have been combined with vascular cells to create functional small-diameter grafts. The main reason for the long-term failure of small-diameter vascular grafts is due to the occlusion (thrombus formation) in the early phase followed by continuous and excessive tissue ingrowths (intimal hyperplasia) in the chronic phase [1]. The seeding of vascular endothelial cells (ECs) onto the lumen of vascular grafts in order to yield a thromboresistant surface and so reduce the high incidence of graft occlusion is one approach that has been proposed to address this problem [2–4]. However, attempts to seed ECs on current vascular prostheses materials is problematic, a major concern being the low number of ECs and proportion of the surface that remains endothelialised on exposure to fluid shear stresses representative of blood flow in the peripheral circulation. It was shown that most EC losses occurred within the first hour of flow and varies...
from 5% to 85% [5,6]. As a result, the plasma proteins will immediately deposit on the denuded areas and subsequently make the foreign materials attractive for platelet adhesion and aggregation [7,8]. Thus any denuded areas on the luminal surface of vascular wall are probable sites for thrombus formation. Occulsive thrombus can quickly lead to graft failure and potentially catastrophic downstream consequences including myocardial infarction and limb ischemia [9].

Due to the low cell number remained, the cell retention and function on vascular grafts has to be improved for clinical applications. It is necessary to grow these grafts in vitro for a period before graft implantation to achieve higher EC coverage and better retention. To address EC attachment and function, cell-adhesive proteins such as fibronectin, vitronectin, laminin, and collagen have been used to modify the surface of scaffold materials [10,11]. Nevertheless, while scaffold materials have been coated with cell-adhesive proteins with some success, some modifications also provide good substrates for platelet adhesion and aggregation [12,13]. Moreover, when exposed to the shear stress in vivo, the ECs bound to some of these biomolecules are not yet stable enough to assure that 100 percent of the scaffold surface is covered by a confluent endothelium.

On the other hand, many biomechanics studies have revealed that shear stress is an important factor in regulating the EC functions [14,15]. ECs subjected to the shear stress are able to convert mechanical stimuli into intracellular signals that affect cellular functions. Ott et al. showed that in vitro long-term shear stress promoted EC adhesion with a reorganization of the acto-skeleton, attachment plaque formation, and extracellular matrix (ECM) production when the cells were seeded on polypropylene hollow fibers [16]. Zhang et al. seeded human umbilical vein ECs onto the lumen of living tissue conduits and increased the shear stress applied on the cells step by step. The results showed that completely confluent monolayer ECs were elongated, and were oriented parallel to the flow direction [17]. Helmlinger et al. found that ECs can respond to different sinusoidal flow conditions by changing their morphologies [18]. In another study, Dardik et al. seeded rat aortic endothelial cells (RAECs) on the inner lumen of polyurethane grafts and exposed to shear stress for 6 days in vitro before implanted into syngeneic rats [19]. The in vitro results showed that immediate graft thrombosis was inhibited and confluent endothelium was presented 3 months after implantation. These findings suggested that in vitro shear stress conditioning of EC-seeded vascular grafts could improve cell retention and function once placed into a hemodynamically active environment. While many studies using different modes of shear forces such as laminar flow and sinusoidal pulsatile flow have provided considerable insights, none of them has applied an actual physiologic pulsatile wave of small-diameter artery to ECs. Actually, most of these flow patterns are very different from the actual physiological small-diameter pulsatile flow which has the pulsatile nature of blood flow and asymmetric shape of the velocity profile [15,20]. The actual physiologic pulsatile flow leading to temporal and spatial variations of the wall shear stress may result in different pheno-types and functions of ECs. Therefore, we believe it is essential to engineer small-diameter vascular grafts under the actual small-diameter arterial pulsatile flow conditions which can function in actual physiologic spatial and temporal dynamics of shear stress.

In our previous studies, ECs demonstrated strong attachment to sulfated silk fibroin nanofibrous scaffolds and proliferated well with high expression of phenotype-related marker genes and proteins under static culture condition [21]. In the present study, a physiological pulsatile flow bioreactor was designed to generate a typical femoral distal pulse wave in vivo. RAECs were seeded on sulfated silk fibroin nanofibrous scaffolds and cultured under three different patterns of flow conditioning, e.g., steady laminar flow, sinusoidal flow, or physiological pulsatile flow. The aim of this study is to find out the best in vitro dynamic culture conditions to generate functional endothelium on sulfated silk fibroin nanofibrous scaffolds for small-diameter vascular tissue engineering. Cell morphology, cytoskeleton alignment, fibronectin assembly, apoptosis, and retention on the scaffolds were investigated and were compared between three different patterns of flow conditioning.

2. Materials and methods

2.1. Preparation of sulfated silk fibroin

Raw Bombyx mori silk fibers were supplied from Suzhou Maoda Textile Co. Ltd. The raw silk fibers were degummed in an aqueous solution of 0.1% (w/v) Na2CO3 with temperature of between 98 and 100 °C. After 0.5 h, the aqueous solution was refreshed; this process was repeated three times until the majority of the sericin has been removed. And then, silk fibroin were dissolved in CaCl2·CH3OH·H2O (mole ratio – 1:2.8) at 78 ± 2 °C with continuous stirring and subsequently dialyzed against distilled water using a SnakeSkin Pleated Dialysis Tubing (PIERCE, MWCO 3500) at room temperature. Finally, the silk fibroin solution was freeze-dried for 24 h to form silk fibron sponges and kept in a vacuum drying dessicator for future use.

The silk fibron sponges were treated in a glass beaker with a solution prepared by gradually adding 10 ml of chlorosulfonic acid (Sigma, USA) to 60 ml of pyridine (Sigma, USA) in an ice bath. The reaction system was gradually heated to 80 °C in a thermostatically controlled bath and kept at constant temperature for 1 h with stirring. After reaction, 200 ml of distilled water was added to the system. Subse- quently, the solution was neutralized by equivalent molar NaOH solution. The insoluble portion was removed by vacuum filtration; the soluble portion was precipitated with 500 ml of ethanol. The precipitate was harvested by centrifugation and dissolved with a small amount of water. And then, it was dialyzed against distilled water using a SnakeSkin Pleated Dialysis Tubing (PIERCE, MWCO 3500) for desalting. After freeze drying, sulfated silk fibron was stored in a vacuum drying dessicator until use.

2.2. Electrospinning of sulfated silk fibroins

Sulfated silk fibron was dissolved in hexafluoro-2-propanol (HFIP; Fluka Chemie GmbH, Germany) to generate a 10% (w/v) solution. Electrospinning was performed with a steel capillary tube with a 1.5 mm inside diameter tip mounted on an adjustable, electrically insulated stand. The capillary tube was maintained at a high electric potential for electrospinning and was maintained in the parallel plate geometry. The capillary tube was connected to a syringe filled with the sulfated silk fibroin/HFIP solution. A constant volume flow rate of 0.8 ml/h was maintained using a syringe pump. High voltage of 15 kV was applied when the solution was drawn into fibers and was collected on rectangular slides kept at a distance of 13 cm from the needle tip. And then, the electrospun nanofibrous scaffolds were treated with 100% methanol for 10 min to induce a β-sheet conformational transition, which results in insolubility in water [21].

2.3. Cell culture

Experiments involving Sprague Dawley rats were carried out in strict accordance with guidelines for the Care and Use of Laboratory Animals of the Beijing Municipal Science & Technology Commission. The protocol was approved by the Ethics Review Committee for Animal Experimentation of the Peking University (SYXK (Beijing) 2006-0025). Surgeries were performed under phenobarbital anes- thesia, and all efforts were made to minimize suffering.

Sprague Dawley rats (100–150 g) were purchased from Department of Laboratory Animal Science of Peking University (Beijing, China), where the use of animal was approved by the local Ethics Committee. RAECs were isolated and cultured by primary explants culture technique as previously described [22,23]. In brief, the thoracic aorta was isolated and adhered to plastic dishes (Corning, USA) while vascular intima downward. The vascular explants were cultured with M199 medium (Gibco, USA), supplemented with 100U/ml penicillin, 100 mg/ml streptomycin, 20% fetal bovine serum (FBS, Gibco, USA) and 50μl/ml heparin (Amresco, USA) in cell incubator at 37 °C with 5% CO2. Fibroblasts contamination was mechanically removed with a sterile scraper and inverted microscope. RAECs were cultured up to 80% confluence, and then were subcultured into plastic dishes (1:2). The cells were identified by their cobblestone appearance and confirmed by staining with a specific antibody to CD31 as described previously [24].

2.4. Flow exposure experiments

A self-designed physiological pulsatile flow tissue engineering bioreactor was used to apply different flow conditions to cultured cells in this research, which consists of a tissue culture system and a pulsatile flow system (Fig. 1A & B). The
The mean shear stress to which RAECs were exposed was 10 dyne/cm², which represent the typical mean shear stress of femoro-distal bypass. A: SLF; B: SF; C: simulated PPF of femoral distal pulse wave.

Fig. 1. (A) Schematic figure of the physiological pulsatile flow tissue engineering bioreactor. (B) Physiological pulsatile flow tissue engineering bioreactor used in this study. (C) The real-time pressure waveforms of different flow patterns generated by a physiological pulsatile flow tissue engineering bioreactor. The time-average shear stress is 10 dyne/cm² which is the peak shear stress of the femoral distal pulse wave.

where \( Q \) is the volumetric flow rate, \( \mu \) is the dynamic viscosity of the culture medium, which is 0.7544 cp in this study, \( w \) and \( h \) are flow channel width and height, respectively. Cells kept on the scaffolds without being exposed to shear stress were used as static control. The culture medium was the same as that mentioned in the cell culture section, except that the concentration of the FBS was 10%. For convenience, steady laminar flow, sinusoidal flow, and physiological pulsatile flow were, respectively, abbreviated as SLF, SF, and PPF in the present research.

At the end of each incubation time (12 h and 24 h), the cell-seeded scaffolds were fixed with 2.5% (v/v) glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH 7.2) for 1 h at 4 °C, washed with Na-cacodylate buffer, and then dehydrated at room temperature in a gradient ETOH series up to 100%. The samples were observed at 800 x magnification using a scanning electron microscope (SEM, JEOL JSM-5600LV, Japan) with an accelerated voltage of 10 kV.

2.5. Cell labeling and detection

Fluorescein diacetate (FDA) molecule probe is well recognized as a viable cell marker. Cells on sulfated silk fibroin scaffolds were rinsed twice with sterile HBSS, incubated in 10 ml of FDA (Sangon biotech, Shanghai, China) working solution (10 μg/ml) at 37 °C for 15 min, and then washed with HBSS twice. Subsequently, cells were observed and photos were taken by an inverted fluorescence microscope (IX71, Olympus Inc., Japan).

2.6. Fluorescent staining of F-actin and fibronectin

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS and then blocked in 1% bovine serum albumin. Primary antibodies to F-actin (Biosen Biotech, Beijing, China) and fibronectin (Boster Biotech, Wuhan, China) were incubated at a dilution of 1:100 with the cells at 4 °C overnight. After washing with PBS, FITC conjugated antibodies (Zhongshan, Beijing, China) at a dilution of 1:100 were added to the cells for 60 min. Nuclei were labeled with DAPI (Sigma). All fluorescent staining was visualized and photos were taken under a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Germany) using a 63 x oil immersion objective lens.

2.7. Cell retention evaluation

After completion of the 24-h culture under each flow conditions, RAECs on sulfated silk fibroin scaffolds were exposed to 1-h continuous steady laminar shear stress (LSS) at 20 dyne/cm² which is the peak shear stress of the femoral distal pulse wave in vivo [14]. Subsequently, RAECs remained on sulfated silk fibroin scaffolds were stained with FDA and visualized by an inverted fluorescence microscope (IX71, Olympus Inc., Japan). Micrographs of cell-covered areas were quantified with a self-written image processing program (mat-lab2010b, USA). The differences of numbers before and after LSS exposure were calculated. The quality of RAEC attachment was measured by the extent of resistance to the peak shear stress. At least three distinct positions of a sample were photographed and calculated.
2.8. TUNEL assay

Cell apoptosis of RAECs cultured on sulfated silk fibroin scaffolds before and after exposure to LS was quantified with terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. TUNEL assay was performed using a commercial kit (In Situ Cell Death Detection Kit, Roche Diagnostics, Germany) according to the manufacturer’s protocol. In brief, Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and then incubated with TUNEL reaction mixture at 37 °C for 60 min in a humidified atmosphere in the dark. Stained cells were visualized and photographed by an inverted fluorescence microscope (IX71, Olympus Inc., Japan). For each sample, at least three randomly selected fields at ×200 magnifications were evaluated. Nuclei with clear green staining were regarded as positive. Nuclei with blue staining represented the total cells. The Apoptosis index (AI) was calculated according to the formula: 100 × the number of positive cells (green)/the number of all visual cells (blue) [25].

2.9. Statistical analysis

Each experiment was repeated independently for at least 3 times. All data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Turkey’s multiple comparison test. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Cell morphology under different patterns of flow conditioning

The electrospun sulfated silk fibroin nanofibrous scaffolds consisted of continuous and randomly oriented nanofibers with diameters ranging from 100 to 550 nm. The SEM micrographs appeared that the nanofibers had a solid surface with interconnected voids among the fibers, presenting a porous network [21]. Morphological changes in shape and orientation of RAECs cultured on sulfated silk fibroin scaffolds were observed at 12 and 24 h, respectively, after exposure to SLF, SF, or PPF (Figs. 2A and B). RAECs under static culture condition showed typical cobblestone appearance and arranged irregularly (Figs. 2A and 3A). Compared with the cobblestone shapes of RAECs under static culture condition, the cell morphologies of RAECs exposed to flow conditions for 12 h were very similar to those of the controls. However, in contrast to the cells under static culture condition, the shapes of the cells exposed to flow conditions for 24 h were quite different. After exposure to different flow patterns for 24 h, the shapes of RAECs on sulfated silk fibroin scaffolds became elongated and their long axis became oriented to the direction of flow. Notably, RAECs under PPF were more elongated and aligned more parallel to the flow direction than that under SF and SLF (Figs. 2G and 3G), which appeared to undergo a more dramatic morphological rearrangement.

3.2. Cytoskeleton rearrangement of RAECs under different patterns of flow conditioning

In this study, fluorescent microscopy was carried out to observe cytoskeletal F-actin filaments stained with antibodies to F-actin. F-actin microfilament in confluent EC monolayers grown on sulfated silk fibroin scaffolds were shown in Fig. 4 for static and flow conditions. Under static culture condition, F-actin microfilaments in RAECs arranged and distributed randomly in cytoplasm (Fig. 4A). Examples of F-actin microfilaments after a shorter exposure time of 12 h under different flow conditions were presented in Fig. 4B, D, and F. With such a short exposure time, a notable change in cellular morphology was not observed with SEM and fluorescence images (Figs. 2 and 3). However, it was observed that the microfilament system was affected. The 12-h exposure (Fig. 4B, D, and F) showed an increased number of F-actin microfilaments located at the periphery of the cell and aligned with the flow field. Fig. 4C, E, and G showed F-actin in response to SLF, SF, or PPF for 24-h exposures, respectively. With the longer exposure time of 24 h, the F-actin microfilaments were mostly aligned with the direction of flow, and more F-actin bundles resided on the cell margin. Notably, for PPF group, there was a much higher concentration of F-actin bundles both at the cell periphery and in the cytoplasm, and the F-actin bundles aligned more parallel to the flow direction than SF and SLF groups (Fig. 4F).

3.3. Fibronectin reassembly under different patterns of flow conditioning

Fibronectin is an adhesive protein and contains the arginine-glycine-aspartic acid (RGD) sequence, known as an integrin binding site, which can improve cell attachment to the scaffolds. RAECs under static culture condition formed thin and sparse fibronectin networks which distributed randomly in intercellular space (Fig. 5A). After exposure to different flow patterns, more fibronectin fibrils were formed and distributed equally on the whole scaffolds.
(Fig. 5B–G). In addition, compared to SLF, the fibronectin fibrillar networks appeared to be thicker and denser under SF and PPF. Notably, after PPF stimulation for 24 h, the fibronectin networks became significantly thicker, denser and highly crosslinked (Fig. 5G).

3.4. Cell retention under different patterns of flow conditioning

Next, we evaluated the effect of different patterns of flow conditioning on cell retention. Vital fluorescent stains of preconditioned specimens confirmed that EC monolayers grown on the sulfated silk scaffold surface were fully vital after 24 h of post cultivation (data not shown). After being cultured under different conditions for 24 h, RAECs were exposed to continuous LSS of 20 dyne/cm$^2$ for 1 h, and then the cells remained on the scaffolds were calculated by FDA staining (Fig. 6). After exposure to LSS for 1 h, cell loss occurred in all grafts. For control grafts under static culture, almost no cells remained (Fig. 6B). Examination of results following different patterns of flow conditioning (Fig. 6D, F, and H) demonstrated a significant improvement in cell retention compared with the control group. Moreover, the cell loss was notably less in grafts preconditioned with PPF as compared with SLF and SF groups. Quantification of these results by image analysis indicated that the ultimate retention of RAECs on sulfated silk fibroin scaffolds was 82.74 ± 2.79% after PPF conditioning compared with 31.38 ± 1.62% after SF conditioning, 15.2 ± 0.6% after SLF conditioning, and 2.55 ± 0.96% after static culture (Fig. 6I).

![Fig. 3. Fluorescence images of RAECs (stained with FDA) cultured on sulfated silk fibroin nanofibrous scaffolds for 12 (B, D, F) and 24 (C, E, G) hours under static (A), SLF (B, C), SF (D, E), or PPF (F, G) conditions. The black arrow indicates the flow direction. Scale bars = 25 μm.](image1)

![Fig. 4. Fluorescence images revealing F-actin rearrangement of RAECs cultured on sulfated silk fibroin nanofibrous scaffolds for 12 (B, D, F) and 24 (C, E, G) hours under static (A), SLF (B, C), SF (D, E), or PPF (F, G) conditions. The black arrow indicates the flow direction. (B–F) Scale bars = 25 μm; (A, G) Scale bars = 50 μm.](image2)
3.5. Cell apoptosis under different patterns of flow conditioning

After being cultured under different conditions for 24 h, RAECs were exposed to continuous LSS of 20 dyne/cm² for 1 h, and then apoptotic cells were assessed by TUNEL assay (Fig. 7). As shown in Fig. 7L, after exposure to LSS for 1 h, no cell apoptosis was found for PPF group. Quantification of the results by image analysis indicated that the ratio of apoptotic cells for PPF group was 0%, which was significantly lower than those of SLF (25.82 ± 2.52%), SF (18.23 ± 2.30%), and static (53.24 ± 5.42%) groups (p < 0.05) (Fig. 7Q).

4. Discussions

The retention of ECs on the lumen of a small-diameter vascular graft in the presence of physiological shear stress is an important prerequisite for long-term patency. The adherence of ECs to the prosthetic surface must therefore be strong enough for them to resist the fluid shear stresses in vivo. It has been demonstrated that in vitro shear stress conditioning of EC-seeded vascular grafts can improve cell retention and function [26]. However, ECs appear to adapt their morphology and function differently after exposure to different flow conditions [18,27,28]. The laminar flow and pulsatile flow conditions which are commonly used in vascular tissue engineering and hemodynamic studies are quite different from the actual PPF in vivo [15,20,29]. EC morphology and function under actual PPF condition should be very different from those under SLF and SF conditions. Therefore, in this study, we investigated the influence of different flow conditions on cell morphology, F-actin microfilament alignment, fibronectin reassembly, cell retention and apoptosis of RAECs cultured on sulfated silk fibroin nanofibrous scaffolds, in order to find out the best in vitro dynamic culture conditions to generate functional endothelium for small-diameter vascular tissue engineering.

An in vitro bioreactor system was used in this study which allowed for SLF, SF and PPF experiments. A representative of human femoral distal pulse wave was simulated and used for PPF waveform to apply to RAECs. Factors were kept with the physiological state of the human femoral distal artery [30]. The frequency of the waveform is set at 1.25 Hz which is a value close to that of the cardiac cycle. The time-average shear stress levels of SLF, SF and PPF are 10 dyne/cm², which is a typical mean shear stress that develops at the proximal anastomosis of a femoro-distal bypass [14]. The simulated flow waveforms were similar to the clinically measured flow waveforms. After 12 h of flow conditioning, the morphologies and orientation of the ECs remained almost unchanged. However, after exposure to flows for 24 h, the shear stress was showed to cause ECs in a confluent monolayer to orient and to elongate from their cobblestone-like, polygonal shape in static culture (Figs. 2 and 3). Notably, ECs under PPF showed a more elongated appearance and aligned more regularly to the flow direction than those under SLF and SF. The results demonstrated that ECs responded differentially to different exposure time and different flow patterns.

The changes with time in cell morphologies appeared to be due to certain structural changes in the cytoskeleton F-actin which are a response to flows [31,32]. F-actin microfilaments are important to the ECs because of their purported roles in cell migration, cell adhesion, and maintenance of cell shape [31]. It is very likely that cytoskeleton remodeling is initiated prior to cell shape and orientation changes [31,33]. In the current study, cultured ECs under static, no flow conditions exhibited a random F-actin microfilaments orientation (Fig. 4). Although a notable change in cellular morphology was not observed for 12-h exposure, it was showed that the microfilament system was affected (Fig. 4B, D, F). After exposure to 10 dyn/cm² for a longer time of 24 h, F-actin microfilaments showed orientation with the major cell axis and with the direction of flow (Fig. 4C, E, G). In addition, there was a higher concentration of F-actin microfilament bundles at the cell periphery and in the cytoplasm for PPF group than SLF and SF groups. The in vivo results provided by White et al. also showed that the F-actin microfilaments were strongly aligned with the flow in the aorta, while in the inferior vena cava there were very few F-actin

![Fig. 5. Fluorescence images showing fibronectin reassembly of RAECs cultured on sulfated silk fibroin nanofibrous scaffolds for 12 (B, D, F) and 24 (C, E, G) hours under static (A), SLF (B, C), SF (D, E), or PPF (F, G) conditions. The black arrow indicates the flow direction. Scale bars = 25 μm.](image)
Fig. 6. Fluorescence images showing cell retention of RAECs cultured on sulfated silk fibroin nanofibrous scaffolds before and after 1-h exposure to LSS of 20 dyne/cm². RAECs were preconditioned under static (A, B), SLF (C, D), SF (E, F), or PPF (G, H) conditions for 24 h. (I) Quantification of the fluorescence images by image analysis indicated significantly improved cell retention of RAECs after PPF conditioning. (Data in mean ± SD, n = 3, * significant difference between PPF group and static, SLF, SF groups at p < 0.05).
microfilaments and with no preferred orientation [34]. The findings in this study indicated that the patterns of flow conditioning and the duration of exposure might be a determinant of EC F-actin microfilaments elaboration and orientation. PPF conditioning could provide EC niche in in vitro studies to mimic physiological conditions in a relevant biomechanical environment. The higher concentration of peripheral F-actin microfilament bundles under the PPF might be crucial for ECs to resist flow shear stress and protect ECs on the sulfated silk fibroin scaffolds from injury. As recently demonstrated by Ethier et al. in a perfusion study, the

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Fig. 7. Cell apoptosis of RAECs before and after 1-h exposure to LSS of 20 dyne/cm² were assessed by TUNEL assay. DAPI was used to locate the nuclei of the total cells (blue). TUNEL was used to locate the nuclei of the apoptotic cells (green). The photomicrographs in E–H and M–P showed DAPI stainings, whereas those in A–D and I–L showed the TUNEL stainings. RAECs were preconditioned under static (A, E, I, M), SLF (B, F, J, N), SF (C, G, K, O), or PPF (D, H, L, P) conditions for 24 h. (Q) The Apoptosis index (AI) of RAECs on sulfated silk fibroin scaffolds after 1-h exposure to LSS. (Data in mean ± SD, n = 3, * significant difference between PPF group and static, SLF, SF groups at p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
cytoskeleton F-actin of ECs played a key role in influencing their flow resistance ability [35], because the peripheral actin bundles could improve the mechanical reinforcement of the ECs.

The microenvironment of an EC in the circulation is under constant mechanical stress, including both fluid-induced cyclical shear stress as a product of the resistance encountered as blood flows through the vessels and longitudinal strain resulting from vessel dilation and relaxation. Cell retention is an even more daunting task for cells used in vascular tissue engineering applications, as the cells will be exposed to in vivo shear stress. In the current study, after exposure to LSS for 1 h, the cell loss was notably less in grafts preconditioned with PPF as compared with SLF and SF groups. As shown in Fig. 61, there was a significant increase in cell retention after PPF conditioning, resulting in 2.63 and 5.44 times higher cell retention than after SLF and SF conditioning, respectively. One reason of how the PPF conditioning results in significantly improved RAEC retention could be explained by the significantly thicker, denser and highly cross-linked fibronectin formed on the sulfated silk fibroin scaffolds (Fig. 5). It is well known that fibronectin, a glycoprotein of the extracellular matrix, plays a major role in cell adhesion, growth, and migration [36]. It serves as an adhesion molecule that anchors cells to ECM mainly via integrins, which mechanically couple the actin cytoskeleton to the ECM via an elaborate adhesion complex [37]. Thus, the regular orientation and denser fibronectin fibrillar networks formed after exposure to PPF can offer more anchors for EC’s binding to ECM and connecting with each other, which might importantly contribute to RAEC adherence and flow shear stress tolerance.

As shown in Fig. 7, the results of TUNEL assay demonstrated that no cell apoptosis was found for PPF group. The ratio of apoptotic cells for PPF group was significantly lower than those of SLF, SF, and static culture groups (Fig. 7Q). The results indicated that PPF stimulation could significantly improve RAECs tolerance to shear stress and effectively prevent RAECs on the sulfated silk fibroin scaffolds from apoptosis. Our results are consistent with the work of Dimmeler et al., which demonstrated that physiological levels of shear stress protected ECs from being driven into apoptosis upon exogenous stimulation with TNFα as well as upon withdrawal of survival factors [38]. This conclusion was also strongly supported by experimental in vivo findings showing that ECs of lesion-prone regions, which were associated with low shear stress or even turbulent blood flow, were characterized by an increased EC turnover rate [39] suggesting a link between low or absent shear stress and EC death. Thus, PPF might contribute to the functional integrity of the EC monolayer and thereby inhibit damage to the arterial wall, which is a key event for initiating atherosclerotic lesion development.

5. Conclusions

In summary, the present data underlined that actual PPF conditioning demonstrated excellent EC retention on sulfated silk fibroin scaffolds, in addition to the alignment of cells in the direction of fluid flow, the formation of F-actin microfilaments and fibronectin in the same direction, and the inhibition of cell apoptosis. Besides the technical problems regarding cell retention, concerns exist as to the ultimate function of these EC on the scaffold surface. The ECs under chronic exposure to an unphysiologic environment could be injured and this would simulate the development of pseudointima hyperplasia after implantation. In view of this point, ECs under actual PPF culture condition should be more advantageous over SLF and SF culture conditions to generate functional endothelium.

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References


