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# Tissue Engineered Small Vessel Conduits – The Anti-Thrombotic Effect of Re-Endothelialization of Decellularized Baboon Arteries: A Preliminary Experimental Study

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Background:** The use of decellularized biological scaffolds for the reconstruction of small-diameter vascular grafts remains a challenge in tissue engineering. Thrombogenicity is an important cause of obstruction in these vessels due to decellularization. Seeding of the decellularized vascular constructs with endothelial cells is therefore a prerequisite for the prevention of thrombosis. The aim of this study was to seed decellularized baboon arteries with endothelial cells and to compare the thrombogenicity to that of decellularized arteries after circulation of blood.

**Material/Methods:** Carotid, radial, and femoral arteries (12 arteries in total) were harvested from 2 *Papio ursinus* baboons. Ten arteries were decellularized. Normal morphology was confirmed in the control vessels. The effect of re-endothelialization was studied in the vessel scaffolds using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Decellularization resulted in vessel scaffolds with well-preserved extracellular matrix and intact basal membranes. Six of the decellularized vessel scaffolds were seeded with viable human umbilical vein endothelial cells (HUVEC). Luminal endothelialization was established after 7 days in a bioreactor and SEM confirmed confluency. Two control, 4 decellularized, and 6 decellularized re-endothelialized vessel scaffolds were studied in an *in vitro* flow chamber using baboon blood.

**Results:** The decellularized arteries showed an absence of endothelial lining, and an intact basement membrane. The seeding process produced a complete endothelial layer on the surfaces of the arteries. After perfusion with whole blood, no thrombi were formed in the control arteries and re-endothelialized vessels. Widespread platelet activation and adhesion occurred in the decellularized vessels despite a relatively intact basal membrane.

**Conclusions:** This study supports the development of re-endothelialized tissue engineered small-vessel conduits.

**MeSH Keywords:** **Blood Coagulation • Blood Vessel Prosthesis • Endothelial Cells • Tissue Engineering • Tissue Scaffolds**

**Full-text PDF:** <https://www.basic.medscimonit.com/abstract/index/idArt/905978>

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## Background

Vascular diseases are responsible for more than 25% of all deaths worldwide [1]. Therapies for vascular diseases often require bypassing or replacement of the diseased vessels with vascular grafts. However, many patients do not have healthy vessels available for grafting due to pre-existing vascular conditions, size mismatch, or available autograft conduits [2,3]. Currently, arteries, such as the aorta or the iliac arteries, are reconstructed using synthetic grafts that are made of expanded polytetrafluoroethylene (ePTFE) or Dacron. These synthetic grafts are also used for reconstruction of small-diameter arteries; however, the patency rates are not favorable because of thrombogenicity and limited re-endothelialization capacity *in vivo* [4]. Some autologous endothelial cell seeding grafts have been implanted with promising results even in very small (4 mm)-diameter grafts that were clinically used [4,5]. The disadvantages, however, were still the use of foreign materials, as well as complicated production and waiting periods of at least 4–6 weeks, for use of these grafts [4,5].

Thus, there is still a worldwide shortage of small-diameter (<6 mm) conduits with sufficient patency rates that can be used to bypass or replace small peripheral diseased arteries [2,3,6]. Autologous arteries are still the criterion standard for vascular replacement due to their inherent physiological properties [7].

Tissue engineered vessels can potentially be used to replace diseased and damaged native blood vessels [8]. Decellularized biological scaffold material from both xenograft and allograft origin can be used in constructing tissues and organs to restore or establish normal function [4,6,7], aiming to develop living autologous grafts with the capacity for growth, repair, and remodelling. Decellularized allograft tissue can also attenuate immune response-related degeneration as a result of chronic rejection by recipients [4]. However, thrombogenicity remains a major concern, as decellularized arteries have no endothelial lining, thus exposing collagen fibers to circulating blood. This direct exposure of collagen results in thrombosis due to platelet adhesion and activation in circulating blood [9]. The absence of an endothelial lining is also associated with accelerated vessel calcification and degeneration [10].

Surface coating of small-diameter grafts with angiogenic growth factors has some promise but does not solve the problem completely due to the inability to form a monolayer [11]. Recently, a more promising approach for construction of small-diameter vascular grafts is the re-endothelialization of decellularized vascular constructs with autologous vascular endothelial cells before implantation [1,10]. Other recent investigations have shown that decellularized scaffolds have no negative effect on cell seeding [2]. The endothelial layer incorporates many of the anti-thrombogenic properties of blood vessels. However,

endothelialization of vascular grafts has been limited due to the cost and availability of reagents, and because it is difficult for endothelial cells to stay attached to the scaffold [11].

The aim of the present study was to re-endothelialize small-diameter (<6 mm) decellularized baboon arteries using cultured HUVECs. Additionally, the re-endothelialized arterial scaffolds were perfused with baboon blood at high shear stress and compared to those of fresh baboon arteries.

Baboon models possess similar hemostatic characteristics to humans. Their coagulation system and platelet behavior closely resembles that of humans, whereas other animal species such as dogs, sheep, and pigs do not. Baboon vascular endothelial cell growth characteristics are also thought to be similar to that of humans. Furthermore, they share about 98% homology to human genes, possess similar protein structures to humans, and reflect the anatomical, physiological, and behavioral make-up of humans [3,12,13].

## Material and Methods

All experiments were performed in accordance with the “Principles of the Laboratory Animal Care” prepared by the National Society of Medical Research, and the “Guide for Care and Use of Laboratory Animals” developed by the US National Institutes of Health, (NIH, revised 1996) and the South African laws for animal welfare. Ethics approval for this study was obtained from the Interfaculty Animal Ethics Committee of the Faculty of Health Sciences of the University of the Free State (NR 17/2014). Two male baboons (*Papio ursinus*) weighing between 10 and 15 kg were anaesthetized with 0.01 ml/kg Rompun (Bayer, Johannesburg, South Africa) and 10 mg/kg Ketamine hydrochloride (SigmaAldich, Johannesburg, South Africa) and euthanized with an overdose of potassium chloride (Bayer, Johannesburg, South Africa). Heparin (17 IU/kg, Pfizer, Johannesburg, South Africa) was given before euthanasia. Twelve medium-sized arteries with diameter smaller than 6 mm and a minimum length of 6 cm were harvested from different regions of the 2 sacrificed baboon bodies. Six arteries were collected from each baboon. The main source of blood vessels was from arteries branching from the aorta: the right (R) and left (L) carotid, R and L radial, and R and L femoral arteries. Ten of the baboon arteries were decellularized. Two femoral arteries were used as controls. Six decellularized arteries (2 from each group) were seeded with HUVECs (Clonetics™ HUVEC systems, Lonza Walkersville, Inc., MD, USA). The remaining 2 decellularized baboon arteries were not seeded. HUVECs were used because they are a readily available cell source for tissue engineering without sacrificing intact vascular tissues. Many other studies have successfully used them for seeding vascular grafts [9]. Furthermore, they are cost-effective and yield large quantities.

## Decellularization

Ten baboon arteries (carotid (n=4), radial (n=4) and femoral (n=2)) were washed with sterile phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Carlsbad, CA) to remove residual blood clots. Decellularization was accomplished using the acid- and detergent-based method [4]. Arteries were decellularized using a combination of 1% sodium deoxycholic acid, 0.05% sodium dodecyl sulphate, and 0.05% triton-X100 at 37°C. Extensive rinsing steps with saline followed this.

A control sample of a circumferential 0.5 cm resection from all explanted arteries was obtained for histology, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) analysis before and after decellularization. We then stored the arteries at 4°C under sterile conditions in PBS containing penicillin (200 U/ml), streptomycin (200 µg/ml), amphotericin B (10 µg/ml), ciprofloxacin (50 µg/ml), and cefuroxime (750 µg/ml) (ScienCell Research Laboratories, Carlsbad, CA).

## Cell culture and seeding

### Laboratory procedures

HUVECs were cultured according to the manufacturer's instructions (Clonestics™ HUVEC systems; Lonza Walkersville, Inc., MD, USA) in a laminar flow cabinet under sterile conditions. The HUVECs were passaged only 3 times for the purpose of this study, to retain the unique function of endothelial cells [14]. To optimize cell culturing, the morphology of the cells was assessed for homogenous cobblestone morphology present throughout the culture, but no pictures were taken of the cells. Cell viability and proliferation rate were determined by the MTT assay (MTT Cell Viability and Proliferation Assay Kit, ScienCell, Carlsbad, CA).

First, the luminal surfaces of the 6 decellularized arteries (carotid (n=2), radial (n=2), and femoral (n=2)) were coated with a solution of 10 µg/ml fibronectin (Human plasma fibronectin, Gibco®, Grand Island, NY) in 1 ml PBS. The arteries were then incubated at 37°C for 45 min to allow the fibronectin to bind onto the ECM. After that, we aspirated the unbound fibronectin and washed the arteries twice with sterile PBS. The decellularized coated arteries were immediately seeded using a bioreactor filled with endothelial growth medium (Clonestics™ EGM-2 Bullet kit medium, Lonza Walkersville, Inc., MD) and kept in a 5% CO<sub>2</sub>/air atmosphere incubator at 98% humidity at 37°C, as previously described [15]. The pH level was maintained at 7.4 by modulating the CO<sub>2</sub> supply. The static environment provided a low shear stress environment during the seeding process. The artery constructs were then washed twice with PBS and supplemented with penicillin and streptomycin to limit any contamination on the vascular grafts.

A density of 2.5×10<sup>3</sup> endothelial cells/cm<sup>2</sup> were used to seed the freshly coated graft surfaces. The EC culture was suspended into the sutured graft within the bioreactor and the air was removed. After substituting ECs, the bioreactor was placed into a biostabilizer (Biegler Medizintechnik GmbH, Mauerbach, Austria) to perform a standardized EC [16]. The bioreactor was rotated to expose the entire luminal surface of the arteries to achieve optimal attachment conditions. After 3 h of seeding, the artery constructs were rinsed with PBS to remove non-adherent cells. The arterial grafts were then maintained in fresh culture medium overnight at 37°C in a 5% CO<sub>2</sub> incubator to allow ECs to grow onto the arteries. This was performed for an additional 7 days, while changing the medium every 48 h. We cut small circular pieces (0.5 cm) from each seeded artery after days 1 and 7 of the seeding procedure to verify the success of endothelialization using SEM analysis.

Cell viability on the scaffold was determined with the same MTT assay kit (MTT Cell Viability and Proliferation Assay Kit, ScienCell, Carlsbad, CA). Two small circular pieces (0.5 cm) from each seeded artery were cut; the cells were trypsinized and then cultured in a 24-well plate for 24 h. HUVECs from the original cell culture were used as a positive control. Only viable cells were counted.

### Perfusion study

Blood samples (50 ml) were collected from 4 healthy baboons in 3.2% sodium citrate and used within 4 h for *in vitro* perfusion. An *in vitro* flow chamber connected to a peristaltic pump was used to provide a closed system, which delivers laminar flow to the arteries. The luminal surfaces of 2 normal, 4 decellularized, and 4 seeded arteries were first washed (2×5 min) with 50 ml of PBS supplemented with penicillin and streptomycin. Then, 50 ml of blood was circulated for 2 h at 37°C with a pressure of 120/80 mmHg through the sutured baboon artery. Calcium chloride (0.6 M) was added to the blood at the time perfusion was started. After perfusion, the vessels were washed once for 5 min with PBS. Small circular pieces (0.5 cm) were cut for SEM and TEM analysis. SEM and TEM analysis were done to assess the morphological differences of arteries before and after the decellularization, re-endothelialization, and the perfusion experiment. TEM analysis was done according to standard diagnostic methods that are widely used for diagnostic purposes. TEM showed the presence and condition of the basement membrane on the luminal surface of the decellularized arteries. Although histology studies were done as well, this is not included in this report, since the SEM and TEM analysis shows more detail.

## Electron microscopy

### Scanning electron microscopy

Each vascular graft tissue sample for SEM was prepared by the Centre for Microscopy at the University of the Free State. All samples were fixed in 2.5% glutaraldehyde (Merck, Johannesburg, South Africa). Tissue specimens were dried using the critical point method (Tousimis critical point dryer, Rockville, MD, USA, ethanol dehydration, and carbon dioxide drying gas) and were metallized using gold (BIO-RAD, Microscience Division Coating System, London, UK; Au/Ar sputter coating @ 50-60 nm). Evaluations were performed with a Shimadzu SSX 550 scanning electron microscope (Kyoto, Japan, with integral imaging). The surface area of each specimen was examined and photographed in different positions. SEM micrographs were used to assess endothelial integrity and to evaluate the quality of the extracellular basal membrane.

### Transmission electron microscopy

Vascular graft samples were fixed in 3.0% glutaraldehyde overnight, post-fixed in Palade's osmium tetroxide, and dehydrated in a graded acetone series [15]. Dehydrated samples were impregnated/embedded in epoxy to facilitate the making of ultra-thin sections for the TEM evaluation. Ultra-thin sections were cut from the sample embedded in the epoxy using an ultra-microtome (Leica Ultracut UC7, Vienna, Austria). After sectioning the samples, they were stained with uranyl acetate and lead citrate. Sections of the leaflet samples were evaluated by using a transmission electron microscope (CM100, FEI, The Netherlands) and photographed using an Olympus Soft Imaging System Megaview III digital camera with Soft Imaging System digital image analysis and documentation software (Olympus, Tokyo, Japan).

## Results

### Decellularization efficacy

#### Transmission electron microscopy analysis

TEM examination of normal baboon arteries showed a normal endothelial lining (Figure 1A) with an intact basement membrane. In a decellularized artery (Figure 1B), the endothelial monolayer was absent. The decellularized artery contained cellular debris and cellular components.

#### Scanning electron microscopy analysis

SEM clearly showed differences between normal and decellularized arteries (Figure 2). The normal artery had a smooth

surface, indicating that there are EC on the luminal surface. The decellularized artery had an intact basal membrane with limited areas with exposed collagen fibers, proving the absence of an endothelial lining.

### HUVECs culture

Although not shown, HUVECs had the typical cobblestone morphology of ECs in growing cultures. Their cell viability in cultures exceeded 90% (Table 1).

### Seeding

#### Scanning electron microscopy (SEM) analysis of seeded arteries

The decellularized arteries supported re-endothelialization (Figure 3). Endothelial cells adhered to the decellularized artery direct after seeding (Figure 3A). Figure 3B shows the proliferation and migration of the ECs on the decellularized artery after 1 day of seeding (indicated by the red arrows), forming an almost confluent monolayer. The ECs had formed an almost confluent monolayer in the middle section of the arterial construct. Seven days after seeding, a complete endothelial layer formed on the surfaces of the arteries (Figure 3C).

#### Viability of seeded HUVECs

Seeded HUVECs showed increased mitochondrial activity with an increased number of cells (Figure 4).

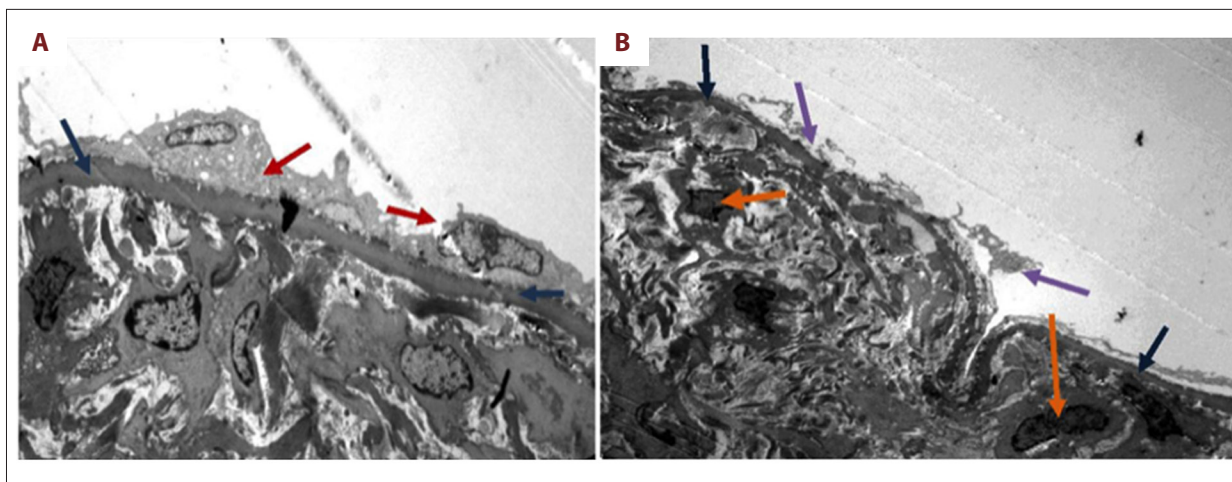
### Perfusion

#### Scanning electron microscopy of perfused arteries

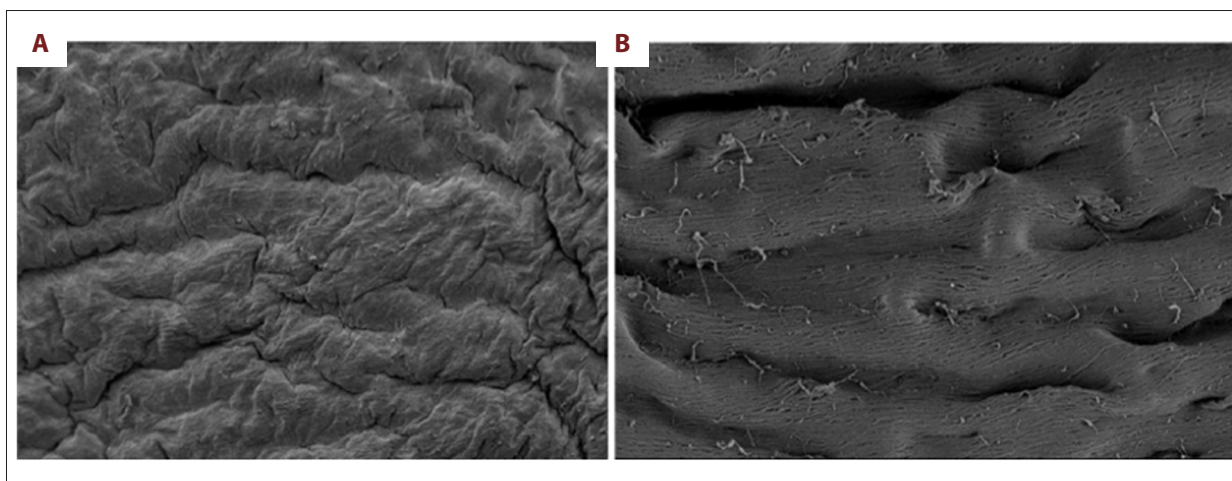
Figure 5 represents SEM images of a decellularized artery (a), a normal artery (b), and a seeded decellularized artery (c) after perfusion with whole blood. The normal artery and the seeded decellularized arteries were devoid of thrombi on their luminal surfaces. There were, however, areas on both with few isolated spots of platelets adhesion. This might be due to possible damage to the endothelial layer or mishandling of the arteries during the seeding process. However, the decellularized arteries (a) had more platelet adhesion and activation on the ECM after perfusion with whole blood, indicating that the decellularized scaffold promotes thrombosis.

## Discussion

The decellularized arterial scaffolds contained some cellular debris but no cells were present, as shown by TEM and SEM (Figures 1, 2). SEM further confirmed the absence of an



**Figure 1.** TEM images of a normal artery (A) and a decellularized artery (B). Both a and b were taken at 1250× magnification. a shows an endothelial monolayer of a normal blood vessel indicated by the red arrows, with an intact basement membrane indicated by the blue arrows (n=2). (B) Shows the missing endothelial layer of a decellularized artery. There was cellular debris on the intimal surface (purple arrows) and ruptured remaining cellular material (orange arrows) throughout the matrix scaffold. It also has an intact basement membrane (dark blue arrows) (n=4).



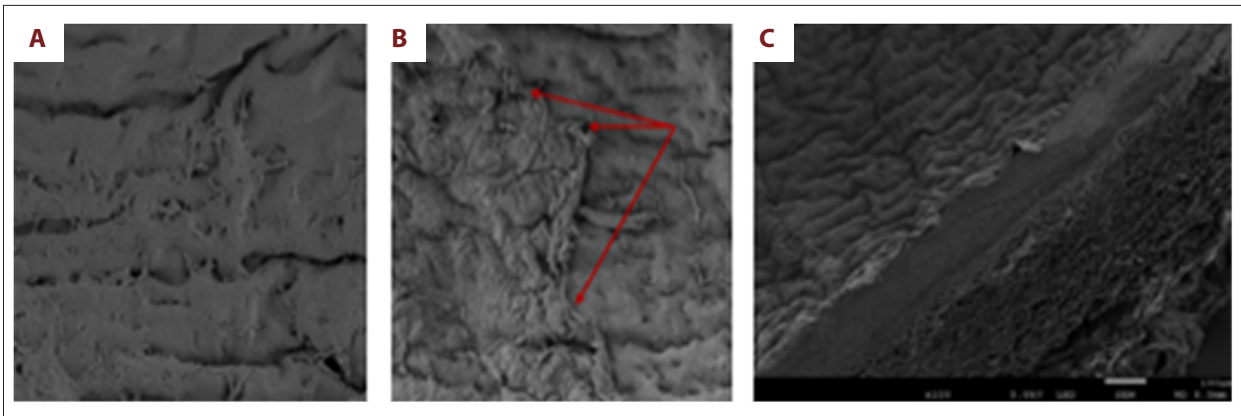
**Figure 2.** SEM images of a normal baboon artery (A) (n=2) and a de-endothelialized baboon artery (B) (n=4). (Images at 1000× magnification).

**Table 1.** Total cell counts and percentage cell viability of the primary culture, after the first passage and after the second passage. Values are expressed as mean (n=2). The total cell count increased with each passage. The percentage viability of the HUVEC cells remained high in the primary culture and increased with each passage.

	Primary culture	First passage	Second passage
Total cell count * 10 <sup>6</sup>	0.97	1.99	2.75
% Viability	94	96	97

endothelial lining. It is important to note that the basement membrane was intact after decellurization (Figure 1). This is an important prerequisite to re-endothelialization because it modulates cell-matrix interactions by supporting cell adhesion, migration, and proliferation during development and regeneration

[6]. Furthermore, a preserved extracellular matrix contributes to maintaining a non-thrombotic environment in graft material if used in bypass surgery [2]. The decellurization process needs to be refined since cellular debris could still be observed. It is, however, important not to damage the basal membrane.

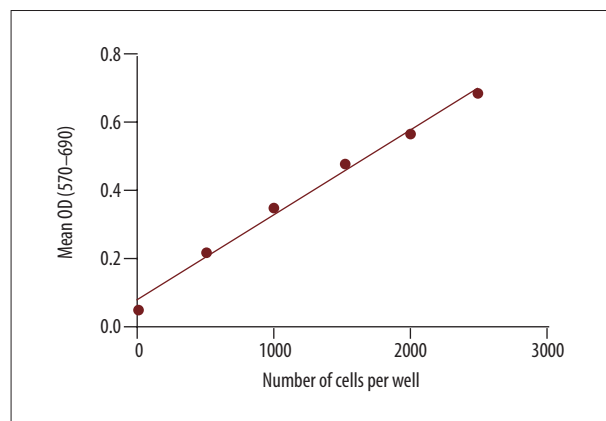


**Figure 3.** SEM images of a seeded decellularized artery. Attached ECs are visible on the scaffold after seeding (A: 1000x magnification, n=3). After day 1, ECs started to form a confluent monolayer and appeared to be migrating towards the direction of the red arrows in image B (B: 450x magnification, n=3). Notice the smooth surface on the seeded area of the decellularized scaffold compared to the rough unseeded area of the artery in image (B). After 7 days of seeding, image (C) indicates a complete endothelial layer on the surface of the arterial sections (magnification 100x, n=3). Notice the smooth surface of the endothelium. The internal elastic lamina separating the tunica intima from the tunica media could be observed from the cross-section. This confirmed the presence of the endothelium.

*In vitro* seeding techniques using cultured autologous cells are required to endothelialize smaller vascular constructs before implantation, since *in vivo* repopulation of smaller decellularized conduits causes thrombosis [15]. We used HUVECs to successfully re-endothelialize the de-endothelialized baboon arteries (Figure 3) because HUVECs are mostly used to obtain ECs for seeding vascular grafts surfaces [11]. Other sources of ECs include endothelial progenitor cells derived from peripheral blood, bone marrow, and umbilical cord vein ECs [5]. We used HUVECs cell cultures because they are readily available commercially. They can also be easily cultured under optimal conditions with a good growth potential. EC cultures were viable and had excellent proliferation capabilities (Table 1).

The decellularized baboon arterial grafts were successfully seeded with HUVECs. The seeded grafts were endothelialized along their entire length from day 1 (Figure 3B), and a confluent monolayer of ECs was observed after 7 days (Figure 3C). Although a slight breakage of the endothelial layer occurred due to twisting during sectioning, a confluent monolayer could still be observed.

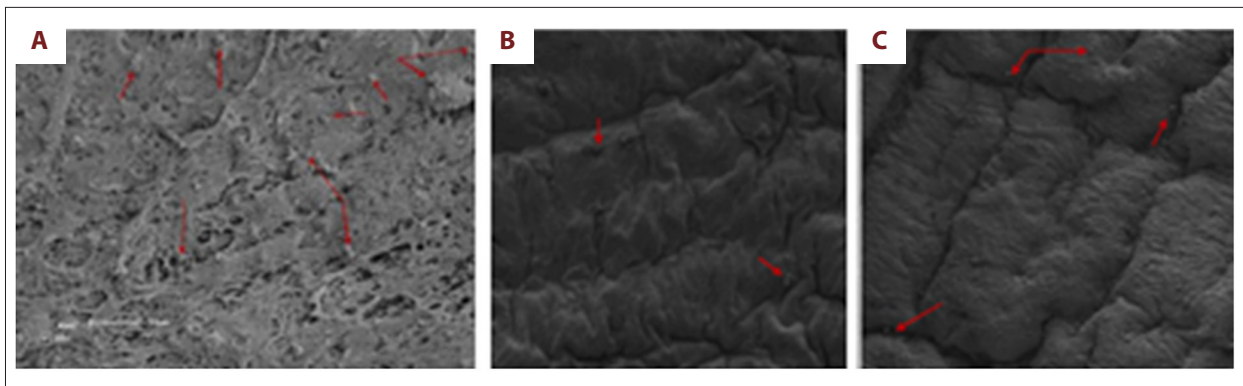
Baboon vessels were used because the hemostatic mechanisms closely resemble that of humans, whereas other animal species such as dogs, sheep, and pigs do not. Their vascular EC growth characteristics are also thought to be similar to that of humans. They also share about 98% homology to human genes and possess similar protein structures [17]. Furthermore, the baboon immunological and coagulation system is closest to that of humans. Baboons show similar fibrinogen level and thrombin time to that of humans. FVIII activity is also similar to humans, and FVIII antigen cross-reacts with



**Figure 4.** MTT assay of seeded HUVECs after 24-h cell culturing from trypsinized small circular pieces (0.5 cm) of the seeded arteries (n=2). The seeded HUVECs showed increased mitochondrial activity with increased number of cells.

human factor VIII antibodies. Response of baboon platelets to collagen ristocetin is similar to humans; however, response to ADP and EPI is slightly reduced and response to arachidonic acid is slightly increased.

The aim of this study was to determine if re-endothelialized, decellularized biological scaffolds could reduce thrombogenicity when compared to the decellularized scaffolds. Decellularized scaffolds promote thrombosis formation due to the absence of an endothelial lining responsible for maintaining an anti-thrombotic surface [17,18]. The perfusion studies support this finding, as unseeded decellularized arteries showed widespread platelet activation during the perfusion experiment



**Figure 5.** SEM results of decellularized (A), normal (B), and seeded (C) arteries after perfusion with whole blood. Image (A) (2000× magnification) demonstrates platelet adhesion and activation on the surface of a decellularized artery (red arrows) (n=4). (B, C) (450× magnification) represent normal (n=2) and seeded (n=4) arteries, respectively. There were few locations (red arrows) of minimal platelet adhesion.

with baboon blood (Figure 5). The seeded arterial grafts and the healthy artery, on the other hand, showed no thrombus formation. However, there were areas where few isolated platelets adhered to the surface. This might have been due to damaged ECs, thus exposing collagen fibers or the underlying basal membrane in those areas. A similar result of platelet adhesion on areas where the EC lining that was not intact is found in the literature [11,17]. In addition, exposed collagen following vascular injury leads to immediate activation of platelets through the activation of the coagulation pathway to seal the wound. It is therefore not surprising that the exposed fibrous structures of decellularized arteries would promote platelet activation.

It is important to note that perfusion did not dislodge the seeded endothelial cells (Figure 5). Endothelialization of vascular grafts is limited by the inability of endothelial cells to remain attached to the scaffold after exposure to flow [1]. The seeding process used in this study was clearly effective in creating an intact endothelial cell lining that withstood high shear stress during whole blood perfusion. It is also encouraging that endothelial viability could be demonstrated after the perfusion study (Figure 5).

### Study limitations

Limitations of the present study include the small number of arteries that were seeded. Since this study was done to prove that baboon arteries can be seeded with human endothelial cells, we did not use many arteries. A follow-up study would be worthwhile with a large number of small arteries, in which autologous endothelial cells or progenitor cells with increased growth potential will be isolated from blood and seeded onto these arteries.

### Future Prospects and Conclusions

HUVECs were successfully seeded on decellularized baboon arteries. The decellularization did not alter the morphology of the extracellular matrix of the arteries and, importantly, the basal membrane remained intact. Endothelialization clearly prevented thrombus formation on the decellularized arterial scaffold surfaces after perfusion with whole blood at high shear rate. Importantly, the 2-h perfusion did not damage the seeded endothelial cells, and these results were similar to those obtained in the perfused control. It is therefore possible to speculate that the findings in a primate model can be extrapolated to humans, and this topic warrants further investigation.

### Conflict of Interest

None.

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