The fate of an endothelium layer after preconditioning

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Background: **A strategy in minimizing thrombotic events of vascular constructs is to seed the luminal surface with autologous endothelial cells (ECs). The task of seeding ECs can be achieved via bioreactors, which induce mechanical forces (shear stress, strain, pressure) onto the ECs. Although bioreactors can achieve a confluent layer of ECs in vitro, their acute response to blood remains unclear. Moreover, the necessary mechanical conditions that will increase EC adhesion and function remain unclear. We hypothesize that preconditioning seeded endothelium under physiological flow will enhance their retention and function.**

Objective: **To determine the role of varying preconditioning protocols on seeded ECs in vitro and in vivo.**

Methods: **Scaffolds derived from decelluarized arteries seeded with autologous ECs were preconditioned for 9 days. Three specific protocols, low steady shear stress (SS), high SS, and cyclic SS were investigated. After preconditioning, the seeded grafts were exposed to 15 minutes of blood via an ex vivo arteriovenous shunt model or alternately an in vivo arteriovenous bypass graft model.**

Results: **The shunt model demonstrated ECs remained intact for all conditions. In the arteriovenous bypass model, only the cyclic preconditioned grafts remained intact, maintained morphology, and resisted the attachment of circulating blood elements such as platelets, red blood cells, and leukocytes. Western blotting analysis demonstrated an increase in the protein expression of eNOS and prostaglandin I synthase for the cyclic high shear stress-conditioned cells relative to cells conditioned with high shear stress alone.**

Conclusion: **Cyclic preconditioning has been shown here to increase the ECs ability to resist blood flow-induced shear stress and the attachment of circulating blood elements, key attributes in minimizing thrombotic events. These studies may ultimately establish protocols for the formation of a more durable endothelial monolayer that may be useful in the context of small vessel arterial reconstruction. (J Vasc Surg 2010;51:174-83.)**

Clinical Relevance: **The importance of ECs toward patency has been demonstrated by the superior performance of endothelialized vein compared with prosthetic vascular graft materials. This article evaluates conditioning protocols for bioengineered vascular conduits to improve endothelial retention. This study describes approaches to improve bioengineered vessels as a potential alternative to conventional prosthetic vascular grafts.**

Vascular graft failure due to thrombosis and intimal hyperplasia is an unsolved problem.[1] Failure of synthetic grafts has been associated with a lack of functionality of vascular cells, mismatch of mechanical properties between host vessel and graft, and poor host remodeling.[2-4] Bioengineered vessels have shown promise in overcoming these issues, as evidenced by their long-term patency and functional arterial segments in preclinical models.[5,6] In native arteries, endothelial cells (ECs) provide a continuous, selectively permeable, thromboresistant barrier between circulating blood and the arterial wall. Moreover, they play important roles in vessel tone, platelet and leukocyte adhesion, and smooth muscle migration and proliferation.[7] A common strategy for minimizing thrombogenicity of bioengineered vessels is the seeding of autologous vascular ECs on the luminal surface of the graft material that will be implanted back into the animal receiving the graft.[8] ECs can be derived from several sources including artery, vein, bone marrow, and blood. Endothelial progenitor cells that circulate in the blood stream and are derived from the bone marrow represent a new potential source of ECs that would be ideal for cardiovascular therapies.[9] ECs derived from endothelial progenitor cells have shown promising results in the development of blood vessel substitutes.[5]

The approach to seeding grafts with ECs has traditionally been performed by either static seeding (solution of cells on graft surface) or static seeding followed by flow preconditioning.[10] Static seeding alone has not shown promising results in either vitro or in vivo studies.[11-13] These disappointing results have been attributed to the lack of a confluent EC monolayer and low EC retention rates in vivo. Improved EC adherence has been achieved by a variety of methods that include optimizing surface coatings, manipulation of the extracellular matrix composition and geometry, improvements in static seeding techniques, and exposing ECs to physiologic mechanical forces.[10,14-16] Among these, exposing ECs to fluid shear stress and cyclic mechanical strain was determined to be one of the most important features in developing a confluent endothelium monolayer with high cell retention rates.

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There currently exist several bioreactor designs and preconditioning protocols in exposing cells to shear stress, pressure, and strain forces.[13,17] Many investigators have been able to show the benefits of bioreactor preconditioning in improving seeding, retention, and function of ECs on a variety of scaffolds in vitro.[18,19] Missing in the majority of these studies is the acute behavior of preconditioned ECs to blood flow. Specifically, the influence of mechanical forces on the ability of cells to resist shear stress, maintain morphology, and interact with circulating blood elements have not been described. These studies are important to establish the appropriate conditions of EC seeding and preconditioning to minimize acute thrombogenic activities and prolong patency of vascular grafts.

The current work investigated the attachment, confluency, and retention of ECs derived from circulating endothelial progenitor cells on a decellularized scaffold using specific preconditioning protocols in both in vitro and in vivo environments. We hypothesized that simulation of physiologic arterial mechanical conditions would improve the adherence and function of ECs lined on a vascular scaffold to create a more durable monolayer of bioengineered vessel.

METHODS

Cell isolation and culture. Endothelial progenitor cells (EPCs) were isolated and cultured as previously described using a proprietary endothelial recovery column system.[20] An extracorporeal EPC purification column was cycled for 1800 mL of blood on adult (aged 4-12 months) female sheep after exposure of the carotid artery and jugular vein Subsequently, cells were eluted from the matrix with trypsin and cultured in EGM-2 endothelial growth media (Cambrex, East Rutherford, NJ) on fibronectin-coated plates. Media was changed at 5 and 8 days after which numerous endothelial colonies emerged and were expanded as mature ECs.

Scaffold preparation. Decellularized porcine arterial segments were prepared as described elsewhere.[5] Briefly, carotid arterial segments were obtained from large pigs from a local abattoir. The blood vessels had an internal diameter of 4 mm to 5 mm and were cut into segments of approximately 50 mm in length. Vessels were washed in deionized (DI) water for 48 hours and incubated in 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA; GIBCO, Invitrogen, Carlsbad, Calif) for 1 hour. The vessels were incubated in decellularization solution (490 mL deionized [DI] water, 10 mL Triton-100X, 3.4 mL ammonium hydroxide) for 3 days in a mechanical rotating shaker at 4°C. The decellularization solution was changed daily. The decellularized vessels were then placed in DI water for an additional 48 hours to wash away any residual processing chemicals from the matrix and then frozen for 24 hours in a −80°C freezer, lyophilized (LABCONCO, Kansas City, Mo), and sterilized with ethylene oxide.

Bioreactor design. We developed a bioreactor system that permitted (1) seeding of acellular substrates, (2) generation and recording of physiologic flow and pressure, (3) maintenance of gases and nutrients in the culture medium, and (4) maintenance of temperature and sterility (Fig 1). The pressure was monitored via a catheter pressure transducer (Millar Instruments, Houston, Tex) and was controlled by adjusting the distal resistance. The flow was controlled by varying the output of a gear pump (Ismatec, Glattbrugg, Switzerland). The output variation of the flow rate was controlled by a computer running LabVIEW (National Instruments, Austin, Tex), which generated steady, sinusoidal, and/or any user-defined waveform with user control over the frequency and time duration for any number of steps. This feature permitted the bioreactor system to steadily increase prescribed flow rates automatically over any duration. The flow was monitored via an ultrasonic flow meter (Transonic Systems, Ithaca, NY) that was positioned upstream of the bioreactor.

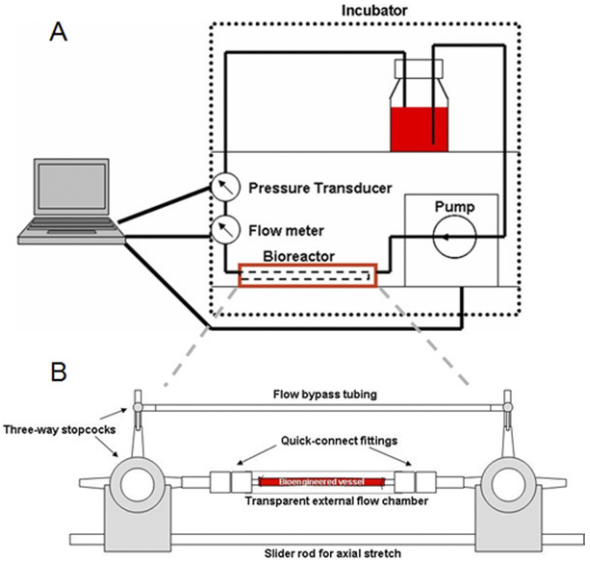

Fig 1. Pulsatile bioreactor system. (**A**) Illustration of the bioreactor system consisting of a computer-controlled gear pump, flow reservoir, ultrasonic flowmeter, and a pressure catheter. (**B**) Illustration of the bioreactor housing unit equipped with a bypass tubing system.

Static seeding and preconditioning protocols. EPC-derived ECs were collected from culture dishes and resuspended in media at a density of 1.0×10^6 cells/mL. In a sterile culture hood, the ethylene oxide sterilized bioreactor parts were assembled. The cells were collected and placed into the lumen of the vessel via three-way valves. The bioreactor was then placed on a custom-built device, which rotated the bioreactor at 0.34 rpm (approximately 3 minutes/cycle) for 2 hours to complete the static seeding procedure.

Following static seeding, the bioreactor was connected to the flow system. Table I illustrates the preconditioning methods for each flow type. The Low SS protocol exposed the cells to a mean wall shear stress value of 1.7 dynes/cm^2 for a duration of 9 days. The High SS protocol exposed the

Table. Detailed bioreactor protocols of the preconditioning methods

	Shear stress (dynes/cm²)				*Pressure (mm Hg)*			
			Cyclic SS				*Cyclic SS*	
Day	*Low SS*	*Steady SS*	*Diastole*	*Systole*	*Low SS*	*Steady SS*	*Diastole*	*Systole*
1	1.7	1.7	1.7	1.7	19	19	19	19
2	1.7	1.7	1.7	1.7	19	19	19	19
3	1.7	3.3	3.3	3.3	19	25	25	25
4	1.7	3.3	3.3	3.3	19	25	25	25
5	1.7	5	3.3	5	19	35	25	35
6	1.7	8.3	6.6	8.3	19	60	46	60
7	1.7	9.9	6.6	9.9	19	74	46	74
8	1.7	11.6	8.3	11.6	19	92	60	92
9	1.7	13.2	9.9	13.2	19	115	74	115

SS, Shear stress.

seeded endothelium to an increasing wall shear stress with a peak value of 13.2 dynes/cm^2 after 9 days. The cyclic protocol induced similar values of shear stress; however, mechanical strain was superimposed to the endothelium layer starting from day 5. Therefore, the ECs were subjected to cyclic shear stress, oscillating pressure, and mechanical strain at a rate of 60 beats per minute. The wall shear rate values induced by the bioreactor system were based on the volumetric flow rates and the viscosity of our fluid. The mean shear stress was calculated by the Hagen-Poiseuille equation:

$$\tau_{mean} = (4\mu Q)/(\pi R^3)$$

in which Q was the mean volume flow rate, μ was the dynamic viscosity, and R was the lumen radius. The assumption was made that the flow through our construct vessels was laminar. The viscosity was measured with a temperature-controlled rotational viscometer (Brookfield, Stroughton, Mass). These tools provided a very accurate measurement of the viscosity ($\pm$ 1% of range) and flow rate ($\pm$ 1 mL/min).

Ex vivo arteriovenous shunt. To expose the preconditioned endothelium to circulating blood, an ex vivo arteriovenous shunt model was developed. Female Dorper Cross sheep between the ages of 4 and 12 months were purchased from a local farm (Mocksville, NC). Sheep were anesthetized and their right (or left) carotid artery and jugular vein exposed through a neck incision. Heparin (100 units/kg) was then administered to the animals prior to the cannulation of the bioreactor tubing to the carotid artery and jugular vein. The bioreactor was positioned via leur fittings between the tubing and primed with saline to ensure no air would be introduced into the vascular system. Three grafts (mean length = 40 mm, mean internal diameter = 5 mm) for each preconditioning protocol were subjected to 15 minutes of blood flow and then rinsed with 60 mL of phosphate buffered saline (PBS). The grafts were then pressure fixed with 2.5% Glutaraldehyde for 48 hours and midgraft sections were prepared for scanning electron microscopy.

Arteriovenous bypass graft model. In order to assess the preconditioned grafts to blood flow in a more physiologic setting, an in vivo arteriovenous bypass graft model was developed. As in the shunt model, sheep were given oral antiplatelet therapy 24 hours prior to any surgical procedure. Sheep were anesthetized and their right (or left) carotid artery and jugular vein exposed through a neck incision. Heparin (100 units/kg) was then administered to the animals prior to the implantation of the seeded bioengineered vessels. The bioengineered vessel was removed from the bioreactor, and a portion of each graft was taken for preimplantation analysis. The grafts (mean length = 50 mm, mean internal diameter = 5 mm) were sewn onto the vein and onto the artery with 6-0 polypropylene suture (Surgipro; Syneture, Norwalk, Conn) in an end-to-side fashion with a standard surgical technique. The direction of blood flow, from artery to vein, corresponded to the direction of the medium within the bioreactor system. After 15 minutes, the bioengineered vessel was removed and rinsed with 60 mL of PBS, and midgraft sections were processed for scanning electron microscopy (SEM) analysis. All the animal care complied with the "principles of Laboratory Animal Care" and received approval from the Wake Forest University Health and Sciences Animal and Care Use committee.

Scanning electron microscopy. To analyze EC morphology and assess the luminal surface of the preconditioned grafts after blood flow, SEM was utilized. Briefly, samples (rings) were fixed in 2.5% Glutaraldehyde solution (Sigma, St. Louis, Mo; 1:10 dilution in PBS) for 48 hours and then cut open and pinned to small strips of Teflon, exposing the luminal surface and dried using a critical-point drying apparatus. Samples were then gold coated and analyzed for endothelial retention, morphology, and surface characterization (SEM Model S-2260N; Hitachi Co, Tokyo, Japan). For each preconditioning method, three grafts were analyzed for SEM. Attached blood elements that included platelets, white blood cells, and red blood cells were counted for each preconditioned methods (n = 9 for each preconditioning group) at a high magnification (1000 X).

Histology and immunohistochemistry analysis. For each set of experiments (bioreactor, ex vivo shunt, fistula) the bioengineered vessels were rinsed with PBS and pressure-perfused fixed in 10% formalin and paraffin embedded. The

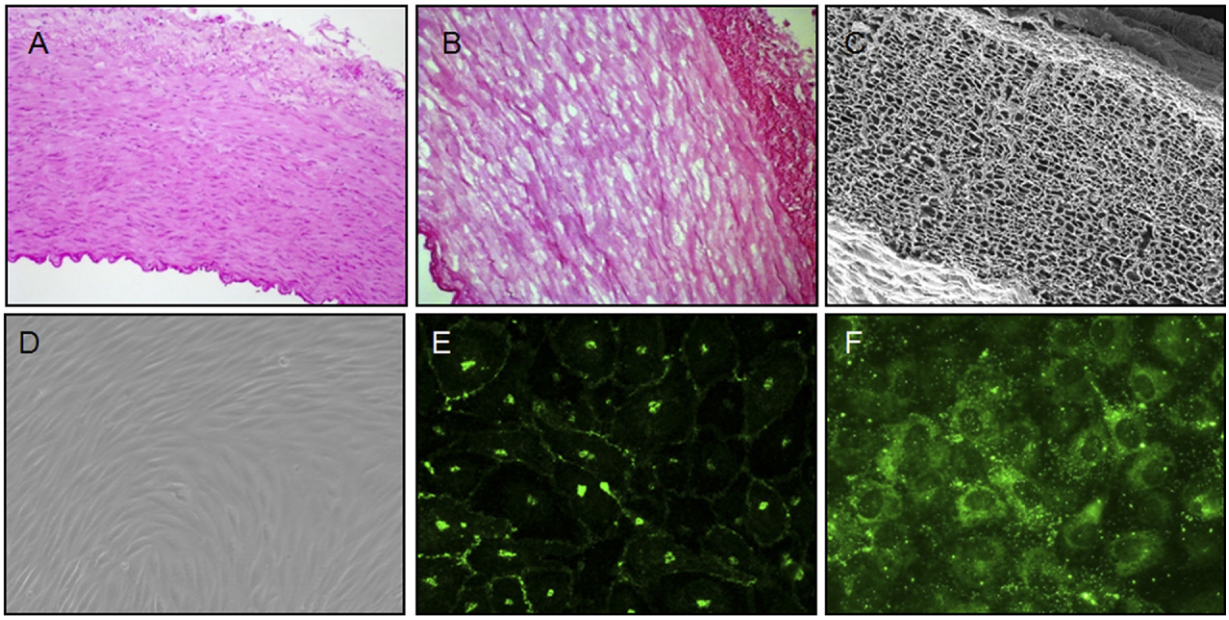

Fig 2. Histological images demonstrating the success of producing an acellular scaffold and the growth of endothelial progenitor cells. Native porcine carotid arteries (**A**) were decellularized in a detergent solution for 72 hours. Both H&E (**B**) and scanning electron microscopy (**C**) confirmed the removal of native vascular cells within the matrix. Autologous endothelial progenitor cells were successfully differentiated in vitro (**D**) and expressed endothelial-specific markers of (**E**) eNOS and (**F**) vWF. *H&E,* Hematoxylin and eosin.

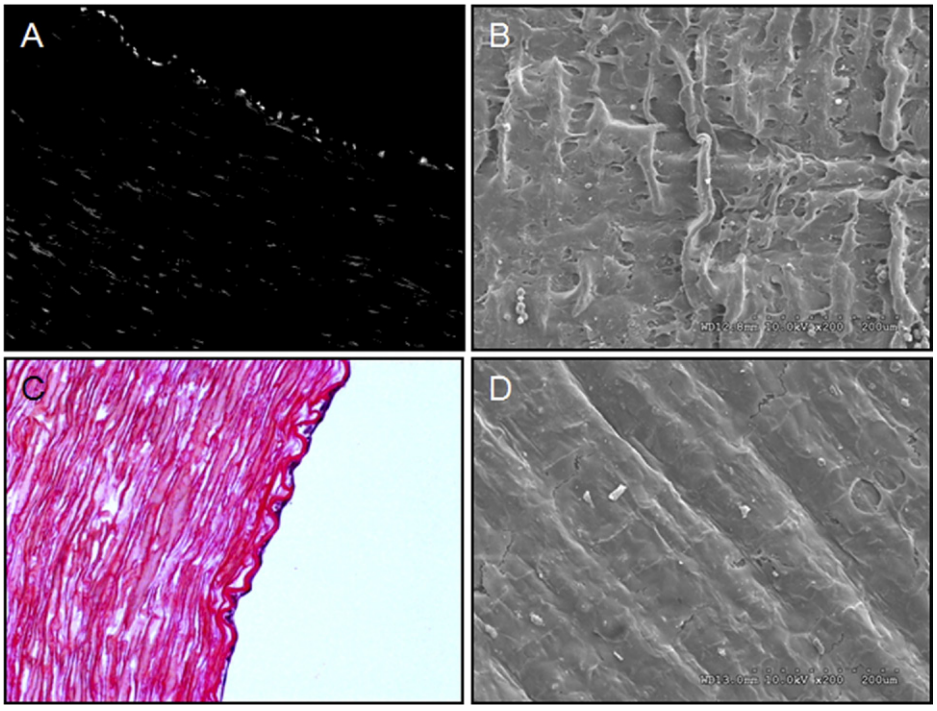

Fig 3. Histological and SEM images of static and low shear stress preconditioned grafts. Grafts statically seeded with autologous EPC did not produce a confluent monolayer as observed with (**A**) DAPI and (**B**) SEM images. Grafts seeded for a duration of 9 days under low shear stress conditions displayed a confluent monolayer in both (**C**) H&E and (**D**) SEM images. *DAPI,* 4′-6-diamidino-2-phenylindole; *EPC,* endothelial progenitor cells; *H&E,* hematoxylin and eosin; *SEM,* scanning electron microscopy.

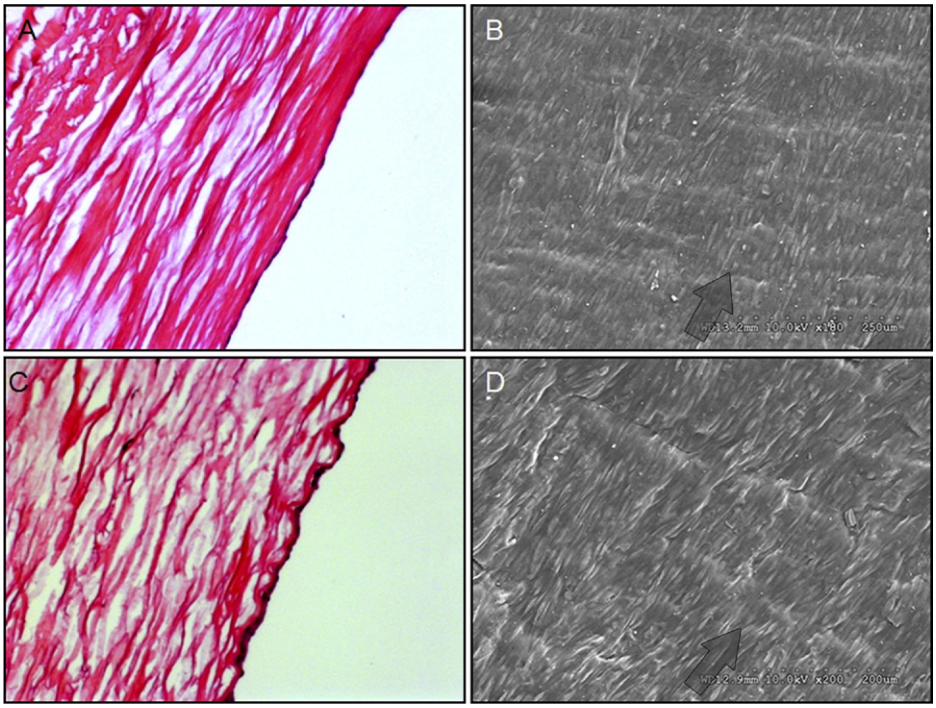

Fig 4. Histological and SEM images of high shear stress and cyclic preconditioned grafts. Grafts seeded for a duration of 9 days under high shear stress condition displayed a confluent monolayer with endothelium alignment as seen with (**A**) H&E and (**B**) SEM images. Similar results were observed with cells preconditioned with the cyclic preconditioning protocol as observed in both the (**C**) H&E, and (**D**) SEM images. Direction of f low is indicated by arrow. *H&E,* Hematoxylin and eosin; *SEM,* scanning electron microscopy.

preimplant segments were immersed in O.C.T. compound (Sakura Finetek; Torrance, Calif) and frozen in liquid nitrogen. Cryostat sections (5 Lm) were analyzed by 4′-6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, Calif). For immunohistochemical analysis, EPCs seeded onto eight-chamber slides were fixed with 2% paraformaldehyde, washed, and incubated with primary antibodies of vWF (DAKO, Carpinteria, Calif) and eNOS (#610297, BD Transduction Lab, San Jose, Calif) using 1:25 and 1:50 dilutions. For staining of Lectin, cells were incubated with biotinylated UEA Lectin (Vector). Primary antibodies or lectin were then localized with FITC anti-rabbit, FITC anti-mouse, or FITC avidin (Vector) at 1:100 dilution.

Western-blot analysis. To determine changes in expression of functional markers due to the mechanical forces induced by the bioreactor preconditioning on the seeded endothelium, quantitative Western blotting analysis was performed. To prepare the cell lysates, grafts were removed from the bioreactor, sliced and pinned open onto Teflon sheets, and rinsed with PBS. Three hundred microL of the lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40] were pipette onto the luminal surface, followed by manual scrapping of the EC. The cell/buffer solutions were agitated for 30 minutes at 4° C and centrifuged at 13,000 rpm for 10 minutes. The supernatant (cell lysates) was then collected and separated by sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membrane. Anti-eNOS (BD) and anti-Prostaglandin I synthase (Abcam, Cambridge, Mass) were used to detect endothelial specific proteins. Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, Md) was utilized to perform densitometry measurements on six samples preconditioned under High SS (n = 3) and Cyclic SS (n = 3) protocols.

Statistical analysis. All results are presented as mean ± standard deviation. Comparisons among the groups were made using a Student's *t* test with JMP software (Carey, NC). The results were considered significant if the *P* value was less than .05.

RESULTS

Decellularization process. In order to obtain a scaffold composed of a biologic framework that would support endothelial cell growth, we decellularized segments of porcine common carotid arteries. These segments possessed an internal luminal diameter of 4 mm to 5 mm and were approximately 50 mm in length. Arterial segments subjected to decellularization and lyophilization maintained their tubular appearance. Hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM) images of the decellularized vessels showed multiple layers of collagenous fibers within the vessel walls (Fig 2). These results demonstrate that the decellularization pro-

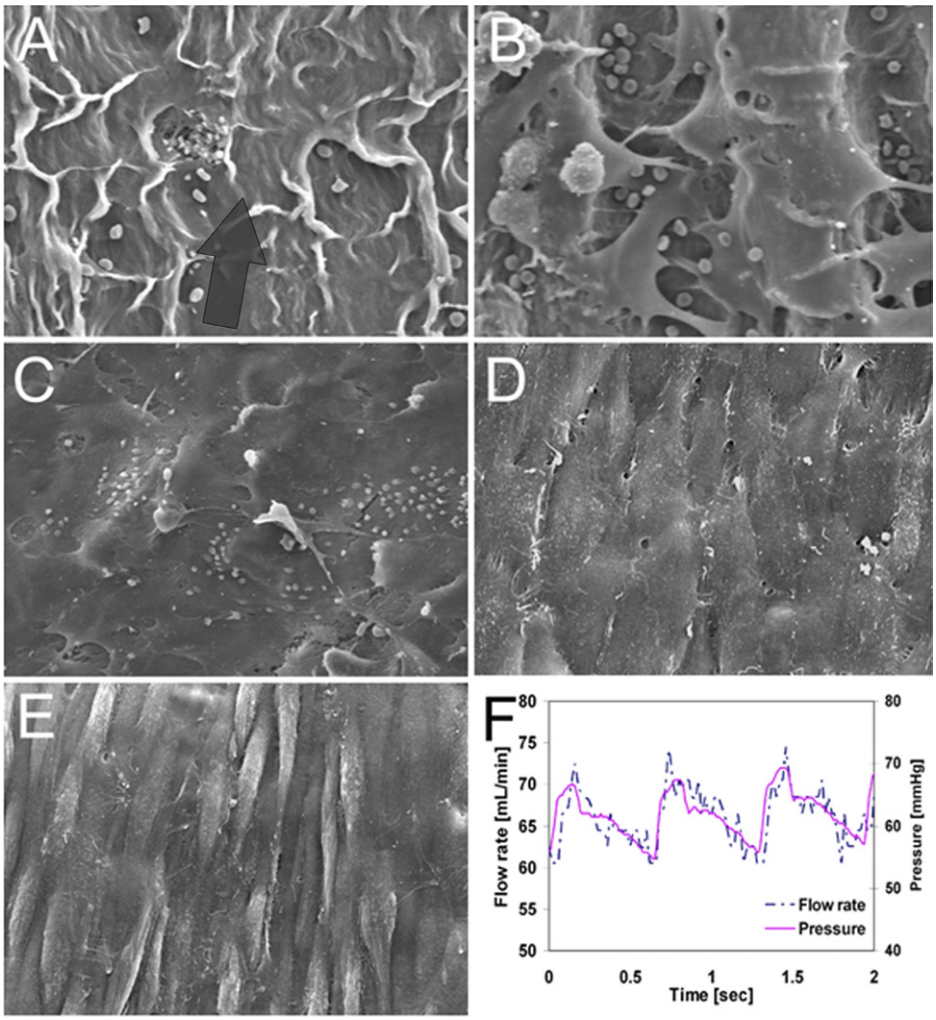

Fig 5. Luminal surface of preconditioned grafts after the ex vivo shunt model. SEM images of the surface for the (**A**) nonseeded, (**B**) static-seeded, (**C**) low shear stress, (**D**) high shear stress, and (**E**) cyclic preconditioned grafts illustrated a lack of adherence of blood elements to the endothelium conditioned under shear stress values > 10 dynes/cm^2. (**F**) Pressure and flow measurements of the ex vivo shunt model. Direction of flow is indicated by the *arrow*. *SEM,* Scanning electron microscopy.

cess removed all the native cells from the vessel, leaving a porous matrix.

Static seeding of EPC-derived EC. Although the decellularized arterial segments maintained an intact tubular structure, a functional confluent EC layer is an essential component for the prevention of graft thrombosis and occlusion. We have previously used sheep endothelial cells derived from EPC to coat decellularized vessels to prevent thrombosis.[5] The EPC successfully differentiated and expanded in culture to form a typical EC monolayer at confluence (Fig 2). Immunohistochemical analysis of EPC-derived EC stained positive for with anti-von-Willebrand factor (vWF), VEGFR2, UEA-lectin, and eNOS. These results indicate that we were able to obtain homogeneous cultures of EPC-derived EC that possess EC phenotype and express specific EC markers.

Fig 3A and 3B show representative examples of results obtained after static seeding. The data demonstrated that the EC adhered to the luminal surface, but it could not develop a confluent monolayer. The seeding efficiency was calculated to be approximately 78%. This calculation was achieved by collecting and counting the unattached cells from luminal volume and comparing it to the original cell count. Doubling the static seeding time to 4 hours displayed similar results and did not significantly alter the seeding efficiency (76%).

Bioreactor preconditioning. Following the static seeding of the endothelium, we exposed the cells to the three preconditioning protocols for a total duration of 9 days (see Table I). The H&E results demonstrated a monolayer of cells on the luminal surface for all three protocols (Fig 3C – Low SS, Fig 4A – High SS, Fig 4C- cyclic). The

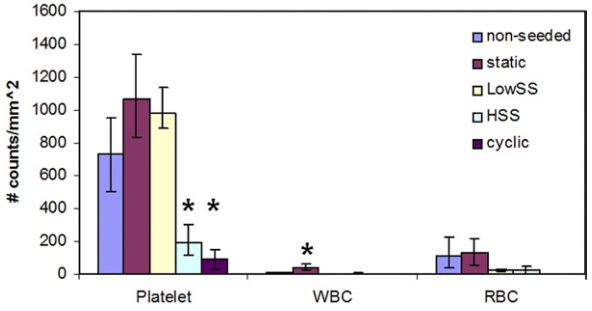

Fig 6. Quantification of blood elements of the luminal surface of preconditioned grafts. Preconditioned grafts were exposed to 15 minutes of blood flow via the ex vivio shunt model. Significant differences (*) were observed between the cyclic and high shear stress group versus the nonseeded, static, and low shear stress groups in regard to platelet attachment. White blood cells (*WBC*) were significantly higher (*) in the static-seeded group as compared with the other four preconditioned groups. Red blood cells (*RBC*) were mostly observed in the nonseeded and static preconditioned groups.

SEM results also demonstrated a fully confluent monolayer for all protocols (Fig 3D, 4B, 4D). However, the cell morphology and orientation differed within the groups. The Low SS preconditioned EC demonstrated a cobblestone-like morphology with no cellular orientation (Fig 3D). The High SS and cyclic preconditioned EC demonstrated an elongated morphology with cellular orientation parallel to the direction of flow (Fig 4B, 4D).

Ex vivo arteriovenous shunt. To determine the interaction of the preconditioned endothelium with circulating blood, an ex vivo shunt model was implemented. The ex vivo shunt model has been used in the past as a tool to assess cell retention and platelet deposition.[21,22] For our studies, the preconditioned endothelium layer was exposed to blood flow for 15 minutes. This exposure time was determined from our own experience in working with these scaffolds in a sheep model and from previous publications demonstrating that in a sheep shunt model, blood element depositions were observed starting only after 5 minutes.[21] Blood flow circulated for a duration of 15 minutes through the grafts with an average flow rate of 65 mL/min and pressure range of 55 mm Hg to 70 mm Hg (Fig 5F). Representative SEM images for each preconditioning method are shown in Fig 5. The data demonstrated that the endothelium layer remained equally intact for the Low SS, High SS, and cyclic preconditioning protocols during the ex vivo shunt, albeit with more pronounced cell alignment in the latter two conditions. Moreover, the High SS and cyclic preconditioning protocols were more resistant to circulating blood elements. Fig 6 summarizes the attachment of blood elements (platelets, leukocytes, red blood cells) to the luminal surface for each preconditioning protocol.

In vivo arteriovenous bypass graft model. Although the ex vivo shunt provided a simple method to expose the preconditioned endothelium to circulating blood, the results suggested that this model did not accurately mimic the mechanical environment of an implanted vascular graft. In fact, our calculations suggest a subphysiologic shear of only 4.5 dynes/cm^2. Energy losses from serial connectors and tubing are likely responsible for the diminished shear stress component. To overcome these limitations and to evaluate the in vivo retention of endothelial cells in a more physiologic setting, preconditioned grafts were sutured between the carotid artery and the jugular vein, creating an arteriovenous bypass graft model. The average flow rates in this model have been measured by our group via duplex ultrasonography to be in the range of 750 mL/min, a significant increase as compared with the ex vivo shunt model. Moreover, the calculated shear stress of 54 dynes/cm^2 is more consistent with physiologic conditions of arteriovenous bypass grafts. Grafts preconditioned under the High SS and cyclic protocols were tested as well as nonseeded and static-seeded. Representative images for each group are presented in Fig 7. The data demonstrated that the endothelium monolayer remained intact and maintained its morphology only for the cyclic preconditioning protocols. No cells could be observed for the static seeding protocol. The High SS preconditioned ECs, could only be observed in patches, with loss of cell orientation. The antithrombogenic properties of the cells could be clearly observed in the cyclic preconditioning protocol.

Expression of functional markers of endothelial function in High SS vs Cyclic SS conditioned grafts. To further understand the effects of bioreactor conditioning of the seeded endothelium, immunoblotting analyses were performed on cells from grafts exposed to the High SS and Cyclic protocols to determine any changes in the expression of eNOS and prostaglandin I synthase (PGIS) on these cells that appear morphologically identical off the bioreactor. eNOS and PGIS trigger the release of nitric oxide and prostaglandin, respectively, which play important roles in the inhibition of platelets and leukocytes to the vascular endothelium. Expression of these proteins has been shown to be stimulated by mechanical forces.[23,24] The data demonstrated a significant increase in expression of eNOS (High SS; 49.65 ± 8.39 OD vs Cyclic SS; 87.64 ± 11.91 OD, $P < .02$] and PGIS [High SS; 8.36 ± 2.87 OD vs Cyclic SS; 90.73 ± 12.36 OD, $P < .001$]) for the cyclic preconditioned cells (Fig 7F).

DISCUSSION

Large, high-flow conduits such as Dacron and PTFE have been used for over 50 years with impressive long-term patency exceeding 86%.[25] Several reports have highlighted the poor endothelial lining of prosthetic grafts even after extended periods of implantation,[26,27] suggesting that in the context of high flow, large-vessel reconstruction, endothelium may be less important for patency. In contrast, the role of endothelium in small-diameter grafts appears to be more substantial, evidenced by the superiority of autologous vein over prosthetic grafts.[28,29] Endothelial cells play a pivotal role in the prevention of platelet adherence, this in turn directly

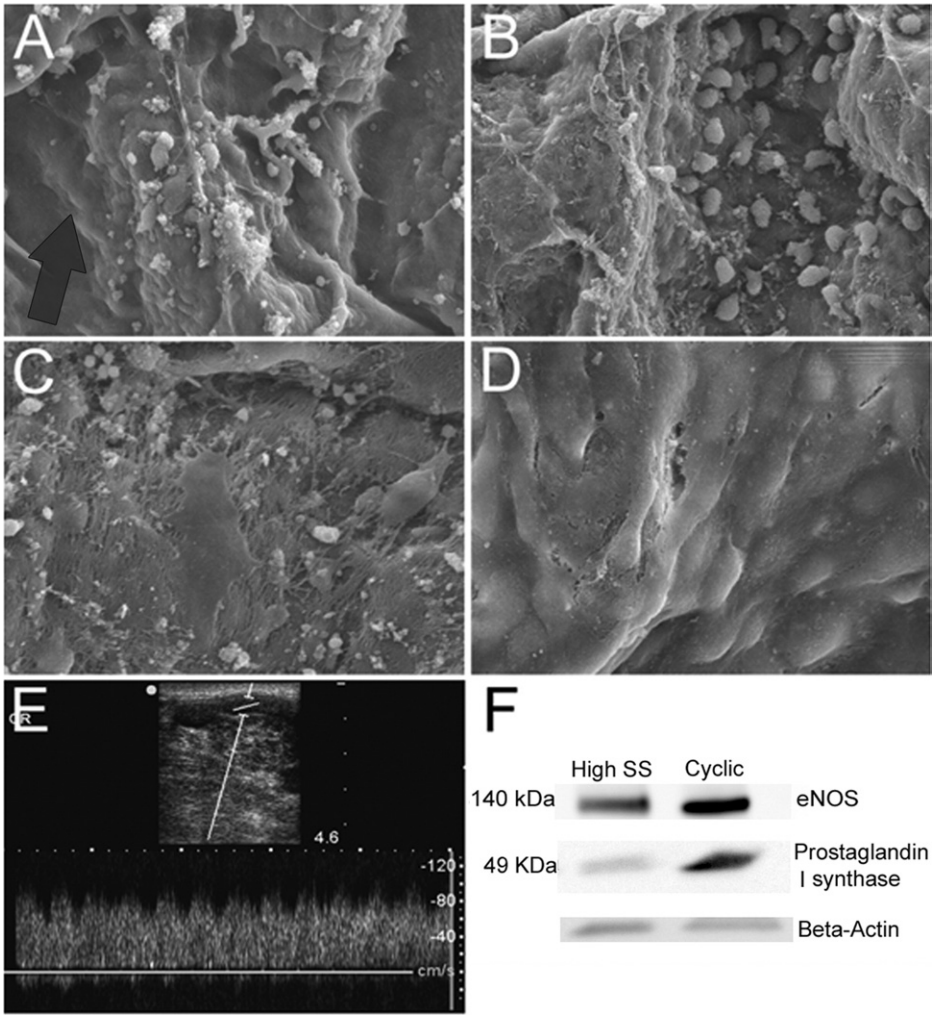

Fig 7. Luminal surface of preconditioned grafts after the in vivo arteriovenous bypass graft model. Preconditioned grafts were exposed to 15 minutes of blood flow in an arteriovenous bypass model. The SEM results displayed loss of endothelial coverage in the (**B**) static-seeded and (**C**) high shear stress groups. Circulating blood elements adhered to the surface of the (**A**) nonseeded, (**B**) static-seeded, and (**C**) the high shear stress preconditioned groups; however, (**D**) the cyclic shear stress-conditioned grafts displayed a high retention rate of endothelial cells with minimal attachment of circulating blood element. (**E**) A representative duplex waveform of the midgraft portion of the arteriovenous graft bypass model. (**F**) Western blot analyses of high shear stress and cyclic preconditioned EPC-derived endothelial cells. Direction of flow is indicated by arrow. *EPC,* Endothelial progenitor cells; *SEM,* scanning electron microscopy.

prevents thrombosis and more indirectly by preventing platelet degranulation,[30] with release of PDGF and TBF-β, for instance, that are believed to be important in late graft failure by intimal hyperplasia (IH).[31,32] Despite these potential advantages, efforts to seed endothelial cells onto prosthetic grafts have been difficult, in part due to electrostatic properties of PTFE that resists adherence of endothelial cells.

The current paradigm, particularly in tissue engineering, is to seed and coat the luminal surface of the graft using autologous endothelial cells.[8] Circulating EPC served as the source for the autologous ECs in this study due to their high expansion capabilities and minimally invasive method of procurement.[5,20] Vascular bioreactors, which can recapitulate a variety of physiologic mechanical conditions to seeded ECs, have been designed as a tool for developing a confluent monolayer. In fact, many researchers have been successful in developing an endothelium layer in vitro on a variety of scaffolds;[5,6,19] however, only a limited number of studies exist on the acute response of the preconditioned ECs to circulating blood in vivo. The important role of flow and shear stress in endothelial cell adaptation has been described in several reports;[33,34] however, the role of cyclic mechanical forces on EC morphology and retention has not been well investigated. To this end, we have developed a bioreactor system that induced three specific mechanical

conditions to investigate EC morphology and retention in vitro and in vivo. Despite an endothelial monolayer in all of the dynamic preconditioning protocols, cell orientation with respect to the direction of flow was only observed in bioengineered vessels experiencing physiologic levels of shear stress, including High SS and Cyclic SS.

Next, we tested our preconditioned grafts in an arteriovenous ex vivo shunt model and an in vivo arteriovenous bypass graft model. The data from the ex vivo arteriovenous shunt experiments demonstrated endothelial adherence for all three dynamic preconditioning protocols with the highest degree of cellular alignment observed in the HSS and Cyclic SS conditioned grafts. The high retention rates of the endothelium were expected, given that the flow through the shunt was measured at approximately 60 mL/min, which corresponds to a relatively subphysiologic mean wall shear stress value of approximately 4.5 dynes/cm^2. However, even with these mechanical conditions, the antithrombotic properties of the varying preconditioning protocols were apparent. The endothelium that was conditioned under High SS and Cyclic SS conditions demonstrated a statistically significant reduction in the attachment of blood elements, particularly platelets, as compared with other conditions.

In order to evaluate a more physiologic degree of shear stress, an in vivo arteriovenous bypass graft model was examined. The average flow rates through these grafts were approximately 750 mL/min, resulting in a mean wall shear stress value of 54 dynes/cm^2. Under these more stringent conditions, a difference between grafts conditioned under High SS and Cyclic SS became evident, with Cylic SS conditioned grafts showing substantially improved endothelial retention and resistance to circulating blood elements. Immunoblotting results similarly reveal an enhancement expression of selected functional markers eNOS and prostaglandin I synthase (PGIS) in cyclic conditioned grafts compared with grafts conditioned with High SS alone. These latter findings represent only a small fraction of the cytokines and adhesion molecules involved in a functional endothelium[36-38] but nevertheless suggest the importance of future investigation into the role of both cyclic shear stress and cyclic circumferential strain on endothelial function.

Several groups have described that in addition to shear stress, that cyclic circumferential strain may provide yet another dimension of endothelial adaptation.[35,36] This strain may be encountered during the rhythmic changes in scaffold diameter that mediate a circumferential stretch and may also modulate endothelial cells independently of the effects of shear stress. These features may explain the differences observed in this study between grafts conditioned with High SS alone versus those conditioned with Cyclic SS.

In summary, ECs have been shown to respond to mechanical forces by changes in morphology and function in vitro. In the present study, we investigated the acute response of preconditioned ECs in vivo for a variety of bioreactor mechanical conditions. This study suggests the importance of including cyclic mechanical forces above and beyond High SS alone during the preconditioning phase in terms of endothelial retention and ability to resist circulating blood elements, such as platelets, under physiologic conditions. In a clinical setting, it is possible that these features may aid in long-term patency of grafts used in vascular reconstruction. Continued optimization of cyclic shear preconditioning may ultimately allow use of bioengineered vessels as an endothelialized alternative to traditional prosthetic graft material in vascular reconstruction.

AUTHOR CONTRIBUTIONS

Conception and design: SY, BT, JB, SS, RG
Analysis and interpretation: SY, BT, JB, SS, RG
Data collection: SY
Writing the article: SY, BT
Critical revision of the article: SY, BT, JB, RG
Final approval of the article: SY, BT, RG
Statistical analysis: SY
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Overall responsibility: SY, BT, RG

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INVITED COMMENTARY

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A major obstacle limiting the success of vascular conduit placement remains the limited ability to obtain endothelial coverage of the graft luminal surface. Among the beneficial functions of a confluent endothelium are thromboresistance, selective permeability, and interactions with circulating blood elements and underlying smooth muscle.

Clinical and laboratory results show that large-diameter high-flow systems tolerate the use of conduits that lack an endothelial lining. That is not the case for small caliber systems and systems that have slower flow.

Conduits with slow blood flow and smaller-caliber conduits perform poorly due to thrombotic complications, possibly due to the lack of nitric oxide and prostacyclin and the longer interaction of the blood elements with the nonendothelialized luminal surface.

This study and others like it focus on defining methods for creating a conduit that has a confluent luminal endothelial lining that tolerates placement in the circulation.

The scaffold, matrix or material of the graft, and the source and conditions under which the endothelial cells are harvested and placed on the conduit are important in determining the degree of success in achieving endothelial confluence and the durability of the endothelial layer when challenged with flow conditions. The authors have demonstrated clearly that, in the short term, the more complex culture conditions of cyclic shear stress produce an endothelial graft lining that functions in a manner superior to that produced by conditions of steady shear stress at low or high levels. Improvement in endothelial function with cyclic shear stress was noted in terms of maintenance of integrity of the endothelial layer and inhibition of adhesion of circulating blood elements to the luminal surface of the graft.

The authors observed by Western blotting increased expression of endothelial nitric oxide synthase and prostaglandin I synthase in the cyclic shear stress conduits compared with the conduits created under other conditions. Defining the role of these or other biologic molecules in conferring favorable graft function will require formal investigation. The functional performance characteristics of the endothelial lining of a conduit likely are the result of many factors, one of which appears to be the type of shear stress used when creating the endothelial monolayer. Other factors may include the scaffold, the origin of the endothelial cells and their culture conditions, both biologic and mechanical.

The ideal conduit for certain applications may also include smooth muscle cells along with endothelial cells to allow for a more durable structure or potentially for biologic molecule delivery via vascularized structures that have genetically engineered cells designed to deliver physiologic regulatory molecules locally or to the circulation. Patterns of molecular expression that may be expected to yield favorable endothelial performance characteristics might possibly include robust eNOS expression and decreased expression of tissue factor and other unfavorable candidate molecules.

Additional work, including longer-term studies and studies in arterial-arterial grafts is required to adequately address the challenge of adequate conduit design.