Endothelial Cell Seeding of a 4-mm I.D. Polyurethane Vascular Graft

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ABSTRACT: We evaluated the extent (luminal coverage) of the endothelial cell (EC) lining/neointimal development and the thromboresistance of electrostatically EC seeded small diameter ChronoFlex-polyurethane vascular grafts. The evaluation consisted of harvesting autologous, canine jugular vein ECs, electrostatically seeding the polyurethane grafts (4-mm I.D., length = 6 cm) with the harvested ECs, implanting the grafts in a canine femoral artery model for four to six weeks, and excising the grafts for histological and scanning electron microscopy evaluations.

Results of the histological evaluation (mid-graft region only) indicated that electrostatic EC seeding led to neointimal development and to minimal to no thrombus formation within the EC seeded grafts. The unseeded control grafts resulted in no neointimal development and substantial thrombus formation on the graft luminal surfaces. Scanning electron microscopy examination demonstrated a mature, confluent endothelium with a ''cobblestone'' appearance on the EC seeded graft luminal surface after six weeks.

We conclude that electrostatic EC seeding enhanced the development of a neointima and reduced the incidence of thrombosis in polyurethane grafts implanted in a canine femoral artery model.

INTRODUCTION

Over 800,000 coronary artery bypass grafts were performed worldwide in the year 2000, utilizing autologous biological blood vessels. In addition, an estimated 30,00,000 patients require femorodistal bypass to alleviate chronic limb ischemia. Despite the widespread use of biological vessels as bypass grafts, these vessels may ultimately fail through two mechanisms: thrombosis and anastomotic hyperplasia.[1] For many years, the search for a suitable synthetic vessel has continued with disappointing results.

The search for a synthetic alternative to autologous biological grafts has been universally filled with disappointment. Heterografts (bovine carotid artery) and homografts (glutaraldehyde-treated human umbilical cord vein) are both prone to long-term biodegradation, dilatation and frank aneurysm formation.[2] Mechanical fatigue due to the lack of collagen cross-links is a postulated mechanism. Cryopreserved saphenous vein, which is commercially available, has yielded poor patency

[1]Mills, J.L., Fujitani, R.M., Tylor, S.M., The characteristics and anatomic distribution of lesions that cause reversed vein failure: a five-year prospective study. *J. Vasc Surg* 1993; 17: 195–196.

[2]Giordano, J.M., and Keshishian, J.M., Aneurysm formation in human umbilical vein grafts, *Surgery*, 1982: 91, 343.

rates likely due to immunologic mechanisms.[3] Another approach was to produce a vascular graft *in vivo* by implantation of a mandril that engenders a surrounding foreign body reaction, yielding a fibrocollagenous tube.[4] Clinical trials with such grafts have yielded consistently poor results with high rates of thrombosis and aneurysmal degeneration.

For large caliber (22–12 mm I.D.) arterial reconstructions, commercially available synthetic grafts [Dacron and expanded polytetrafluoroethylene (e-PTFE)], offer good outcomes. Long-term results of synthetic grafts for replacement of the thoracic and abdominal aorta, arch vessels, iliac, and common femoral arteries for either aneurysmal or occlussive disease are generally acceptable, although graft infection, occlusion and dilatation continue to be important clinical problems. Unfortunately, synthetic grafts have proven unfavorable as small caliber (<5 mm I.D.) arterial substitutes. In these demanding, low blood flow environments, the primary factor influencing long-term patency is the type and quality of conduit used, with other patient variables (e.g., clinical indications, outflow resistance, site of distal anastomosis, comorbidities) serving as important modifiers.

Long-term patencies must be improved in order to enable successful clinical application of synthetic small diameter (<5 mm I.D.) vascular prostheses. One highly attractive approach to improve patencies involves endothelial cell (EC) seeding of synthetic grafts [1]. Historic EC seeding techniques yield a low number of adhered ECs prior to implantation, and, upon implantation, the seeded vascular prostheses suffer substantial (up to 70%) EC loss upon exposure to fluid shear stress [2]. Therefore, to enhance long-term graft patencies, advances are needed in EC seeding techniques. These improvements include: (1) harvesting of greater numbers of autologous ECs and (2) increasing the initial number of ECs seeded to the graft, as well as their maturation, prior to implantation [3]. The electrostatic EC seeding technique focuses on the second issue, namely, enhancement of EC adhesion and maturation phase during graft seeding prior to implantation of the vascular prosthesis. This is accomplished by temporarily altering the prosthetic graft luminal surface charge to a more positive state. The optimization of this seeding technique with expanded e-PTFE resulted in an increased total number of adhered ECs.

[3]Faggiolo, G.L., and Ricotta, J.J., The role of cryopreserved vein graft allograft in infrainguinal reconstruction. In *Advances in Vascular Surgery*, (Whittemore, A.D., ed.) 1995: Vol. 3. Mosby Year Book, St. Louis.
[4]Sparks, C.H., Silicone mandril method for growing reinforced autogenous femoropopliteal graft in situ. *Ann. Sur.* 1973: 177,293.

Furthermore, the total number of ECs adhered in the flattened phase was also increased [4–7]. The mature, flattened phase is important in minimizing the loss of ECs upon exposure to fluid shear stress. Flattened phase ECs not only have an increased number of contact regions with the synthetic graft material, but they are also in a stage best suited to allow streamlining of blood flow around the cell.

Much of the historic work on EC seeding has been driven by the perceived need to increase the total number of ECs adhering to the grafts. The electrostatic seeding technique focuses not only on increasing the total number of adhered ECs but also on altering the stage, or morphologic phase, of the adhesion via a temporary charge alteration of the graft material luminal surface. The ECs initially adhere as a spheroid with a point attachment to the substrate. Over time, the ECs mature, and their morphological maturity progresses from the spheroid to a discoid and, finally, to a completely flattened shape. Again, this maturation is important for two reasons: first, the flattening and spreading of the cells increases their region of contact with the substrate, leading to better adhesion when subjected to fluid shear stress and, therefore, less cell loss; second, the increased adhesion allows for streamlining of blood flow around the cells and, therefore, through the implanted graft section. The increased spreading during EC seeding is an important aspect of the process. A study by Pratt et al. [8] has shown that EC adhesion to a vascular graft exposed to flow conditions is dependent on the EC–surface interaction and the time allowed for adhesion and spreading prior to the initial exposure to fluid shear stress. They suggest that ECs that are not tightly adhered to the graft surface are washed away when implanted and exposed to fluid shear stress. Furthermore, it has been reported that as many as 30% of the seeded ECs are sheared away from the implanted grafts over the first 30 min of implantation with 3.7% lost per hour during the period from 30 min to 24 h [2].

Many investigators have attempted to increase the number of ECs adhered, as well as the degree of adhesion (morphological maturity), by placing adhesive proteins such as fibronectin on the graft lumen to act as a ''glue'' for the ECs [9–11]. The ''glue'' does enhance EC adhesion during seeding, but, if the surface is not completely endothelialized or ECs are lost upon flow exposure, this same ''glue'' attracts platelets. Thus, the ''glue'' results in a thrombogenic surface, defeating the purpose of EC seeding. The advantage of the electrostatic seeding technique is that the enhanced EC adhesion is induced by a temporary positive surface charge or ''temporary glue'' on the negatively charged graft luminal surface. When the seeding

process is complete, the graft lumen rapidly ($\sim 10^{-8}$ s) reverts back to the original highly negatively charged surface [12]. Thus, any non-endothelialized surface or exposed surface due to EC losses upon implantation is non-thrombogenic due to its high negative surface charge.

After conceiving of the concept of electrostatic EC seeding and fabricating a prototype apparatus [5], the first step was to optimize the procedure. The ECs used in the optimization study were Human Umbilical Vein Endothelial Cells (HUVECs) (Clonetics Corp., San Diego, CA). These cells were seeded onto 4-mm I.D. GORE-TEX® standard wall grafts. The purpose of this optimization was to find the applied voltage/seeding time combination that produced the maximum number of adhered ECs, maximum morphological maturation of the cells, maximum viable graft surface coverage, and minimal cellular membrane damage. The applied voltage/seeding time combination of + 1.0 V and 16 min fit the a priori optimum conditions [6].

Using the optimum electrostatic EC seeding conditions (+ 1.0 V/16 min), an *in vitro* flow loop evaluation comparing electrostatic versus gravitational seeding techniques was conducted by exposing HUVECs seeded on 6.0 cm segments of 4-mm I.D. e-PTFE grafts to a shear stress of 15 dynes/cm^2, a value comparable to the average shear stress in the femoral artery [13]. The control graft (0.0 V/16 min) exhibited a 30% loss of ECs at 30 min, which was significantly ($p<0.05$) different from the control prior to shear stress exposure. The electrostatically seeded grafts (+ 1.0 V/16 min) showed no significant ($p<0.05$) loss of ECs from the graft after either 30 or 120 min of exposure to shear stress. Furthermore, the electrostatically seeded grafts had more than 2.4 times ($p<0.05$) the amount of ECs than the gravitationally seeded grafts after 120 min of exposure to 15 dynes/cm^2 [7].

An *in vivo* evaluation [14,15] of the electrostatic EC seeding technique has been conducted to demonstrate the thromboresistance of seeded e-PTFE grafts. This *in vivo* (canine) evaluation utilized autologous jugular vein ECs harvested using a 0.2% collagenase solution. These ECs were electrostatically seeded (+ 1.0 V/16 min) onto 4-mm I.D. e-PTFE grafts ($L = 6$ cm) and then implanted immediately into a femoral artery along with a contralateral untreated control graft ($n = 10$) for six weeks. Aspirin therapy (325 mg/day) was administered for the first two weeks post-operation and discontinued for the remaining four weeks. The electrostatically seeded grafts resulted in a 100% patency rate, while the control grafts had a patency rate of 70%. The electrostatically seeded grafts experienced minimal graft encapsulation; the control grafts demonstrated a high degree of tissue ingrowth.

Internal visual and histological examinations of the electrostatically EC seeded graft luminal surfaces revealed minimal to no thrombus formation with extensive neointimal development, while the control grafts demonstrated minimal pannus EC ingrowth and extensive thrombus formation on all luminal surfaces. Thus, the electrostatically seeded grafts demonstrated high thromboresistance despite discontinuation of the aspirin therapy.

The clinical significance and potential value of this electrostatic EC seeding technique is supported by the results of the *in vitro* and *in vivo* studies utilizing e-PTFE (as discussed above) and polyurethane (CardioTech) (as presented in this report) vascular grafts. The results of the *in vitro* evaluation have demonstrated that the morphological distribution of the adhered ECs tends more toward the flattened phase of adhesion with the electrostatically seeded grafts and more toward the spheroid phase of adhesion for the control grafts under identical conditions. It is thus inferred that this ''temporary glue'' technique leads to increased contact regions between the EC and the graft material. In addition, the total number of ECs adhered increases [6]. This combination of ''temporary glue'' effects satisfies several of the key requirements for improving the clinical application of small diameter vascular prostheses. Another major advantage of this electrostatic technique over other seeding techniques is that the enhanced EC spreading, increased contact regions (speculative), and increased total number of adhered ECs is accomplished in a practical time period (16 min) as compared to hours of culture time. Moreover, when the electrostatic charge is removed from the graft material, the surface reverts back to its initial highly negative charge, which reduces the chance for initiation of an acute thrombus. This significant thromboresistance has been demonstrated by the *in vivo* (canine model) evaluation discussed above. Consequently, it is speculated that the enhanced EC adhesion allows time for the seeded ECs to proliferate and to promote complete healing without vessel occlusion. Studies have shown that the intimal hyperplasia caused by smooth muscle cell proliferation slows down as EC healing progresses, except in the anastomotic region [16]. No data on the effect of electrostatic EC seeding on anastomotic hyperplasia is available at this time (future phase of research). Thus, the distinct possibility exists that all caveats of the electrostatic seeding technique may lead to a reduction in complications currently associated with the clinical use of small diameter (<6 mm I.D.) synthetic vascular prostheses.

The specific aim for this study was to determine the thromboresistance in an *in vivo* canine femoral artery model of the electrostatically

seeded ECs on the CardioTech 4-mm I.D. vascular graft in comparison to an untreated, bilateral control. The *null hypothesis* for this *in vivo* study was that the electrostatic EC seeding technique does *not* affect the degree of thrombus formation on the CardioTech 4-mm I.D. vascular graft after a six-week implantation. Additional histological data obtained included the extent (luminal coverage) of the EC lining. Prior to this *in vivo* study, a limited scope preliminary evaluation of electrostatic seeding of the CardioTech vascular graft material was performed to determine the optimum electrostatic seeding condition in terms of applied voltage to the prototype seeding apparatus reconfigured to accommodate the 4-mm I.D. CardioTech vascular grafts.

MATERIALS AND METHODS

Electrostatic EC Seeding Apparatus

The evaluations of the electrostatic EC seeding technique were conducted using small diameter (4-mm I.D.) CardioTech graft segments. The prototype apparatus used to induce the positive graft lumen charge was modified (external conductor machined) to allow placement of the CardioTech graft segments and is shown in Figure 1 (U.S. Patent Number 5,714,359). A filling apparatus is attached to the base of the

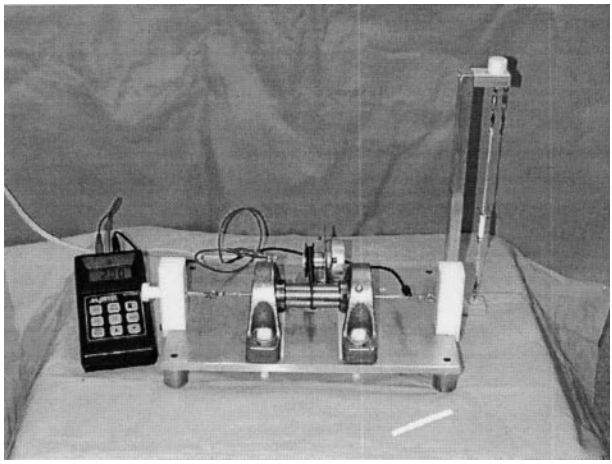


Figure 1. Photograph of the prototype electrostatic EC transplantation device.

seeding apparatus and is used to hold the internal conductor within the graft during the filling of the graft with ECs suspended in Dulbecco's phosphate buffered saline (DPBS). The prototype seeding apparatus consists of an outer cylindrical conductive 303 stainless steel rod. This rod is bored out to allow for placement of the 4-mm I.D. graft specimen within it. The bore size is slightly larger than the external diameter of the 4-mm I.D. vascular prosthesis. The external conductor is held in place by two pillow blocks, which also allow for rotation of the external conductor. The internal conductor is composed of a 0.65 mm diameter (AWG 22) GORE-TEX® coated wire, Sylgard® (DOW Corning Corp.) plugs, and ball bearing, swivel end terminals. The Sylgard® plugs (soft polymer) are used to seal the graft segment in place on the internal conductor, as well as maintain the GORE-TEX® coated wire through the center of the graft during the seeding procedure. Electrical potential is applied across the two conductors by a voltage calibrator (Davis Instruments) with a range of 0–20 ± 0.01 V. These components together induce a **temporary positive surface charge** on the lumen of the graft (dielectric material). The internal conductor is held taut and centered within the graft by two end supports. Eyehooks are in place on the end supports; a spring on one eyehook allows for the removal of any slack in the internal conductor. The rotation of the apparatus is accomplished with a motor driven system: a rubber belt connects the motor to the external conductor. The rotational speed of the graft during EC seeding is 1/8 rpm [5].

EC Seeding/Seeding

Endothelial cell seeding was accomplished by first placing the internal conductor through the graft specimen. The bottom end of the graft segment was tied to the internal conductor (Sylgard® plugs) with 2-0 Silk suture to prevent leakage of the EC–DPBS suspension. The graft was filled (slowly to allow air bubble escape) with the EC–DPBS suspension using a syringe, and the top end was sealed with 2-0 Silk suture. The internal conductor with the filled graft was then placed through the external conductor. The internal conductor was secured to the end supports by the ball bearing, swivel end terminals, and the entire apparatus was rotated at 1/8 rpm. The seeding voltage (+ 2.0 V, as determined by an *in vitro* preliminary study) was then applied across the inner and outer conductors for the seeding time (16 min). The positive lead from the voltage calibrator was attached to the external conductor, and the negative lead was attached to the internal conductor.

METHODOLOGY – PRELIMINARY *IN VITRO* STUDY

EC Culture

The HUVECs (Clonetics Corp.) used in this study were from a single donor (~500,000 HUVECs/amp) and cryopreserved until experimentation. The HUVECs were cultured at 37°C, 5% CO_2, and constant humidity through two passages in endothelial growth medium (EGM). The EGM was composed of Medium-199, 10% fetal bovine serum (FBS), 50 Units/mL heparin, 20 μg/mL endothelial cell growth supplement (Collaborative Biomedical Products), 20 mM L-glutamine, and a Penicillin–Streptomycin–Fungizone® mixture (Whittaker M.A. Bioproducts, Inc.; 1.25% (vol.)), composed of 10,000 Units penicillin/mL, 10,000 Units streptomycin/mL, and 25 UG amphotericin-B/mL.

Cell Harvest, Resuspension, and Electrostatic Seeding

The harvest of HUVECs at the second passage from the tissue culture flask began by removing the EGM and rinsing the cell monolayer with 5 mL of HEPES Buffered Saline (Clonetics Corp.). Four milliliters of 0.025% trypsin/EDTA (Clonetics Corp.) at 37°C was added to the flask, and, after 7 min, 4 mL of trypsin neutralizing solution (Clonetics Corp.) was added. The resulting HUVEC suspension (~8 mL) was transferred into a 15 mL centrifuge tube and centrifuged (@110 g) for 8 min. Following removal of the supernatant, the HUVEC pellet was resuspended (5×10^6 HUVECs/mL) in DPBS containing antioxidants (1.3×10^{-3} M dimethyl sulfoxide, 1×10^{-4} M sodium ascorbate, and 3.6×10^{-5} M glutathione) used to protect the cellular membranes from damage due to free radicals [17]. Electrostatic EC seeding was conducted as previously described at 0.0, +1.0, +2.0, and +5.0 V applied to the prototype apparatus for a constant 16 min seeding period. The length of graft segments utilized for the *in vitro* study was 3 cm.

SEM Examination

The mid-graft specimens (Formalin-fixed) were dehydrated with a series of acetone–water solutions, then critical point dried, mounted on aluminum stubs, and sputter coated with gold. The samples were then examined utilizing a scanning electron microscope (JSM-820; JEOL, Ltd.). The purpose of this scanning electron microscopy (SEM)

examination was to enable a detailed, visual examination of the luminal surface (EC seeding density and morphological maturation).

METHODOLOGY – PRELIMINARY *IN VIVO* STUDY

Autologous EC Harvesting

All procedures of animal care and use were approved by the Virginia Commonwealth University/Medical College of Virginia Institutional Animal Care and Use Committee and complied with standards issued by the Public Health Service and the American Accreditation of Laboratory Animal Care (AALAC). Animal preparation for surgery and sterile surgical techniques followed standard protocols. The canines used in this study were female adults weighing between 25–30 kg (the additional four-week study ($n = 2$) used 30–35 kg male canines). Complete blood work (blood count, serum chemistry, and coagulation) was performed on each canine prior to admittance to this study; only canines possessing normal coagulative properties were used to reduce the likelihood that a hypercoagulable canine would skew the results. Conversely, a hypocoagulable canine could possibly experience excessive bleeding after graft implantation and cause a premature end to the *in vivo* evaluation.

One day prior to surgery, the canines began an anti-coagulation regimen of aspirin (325 mg/day), which was continued for two weeks post-implantation. *Note*: The authors are aware of the vulnerability associated with the discontinuation of the anti-coagulation regime at two weeks post-implantation. In a clinical setting, the aspirin (anti-coagulant) regime would be continued for the life of the implant. The rationale here, however, is to expose the EC seeded grafts to an active clotting environment (normal platelet function) to allow realistic determination of enhanced thromboresistance due to electrostatic EC seeding. The experience of the authors is that, if aspirin is used throughout the implantation period, a minimal or zero thrombus formation will be seen on any (including control) of the implanted grafts over the four- or six-week period. The antibiotic Cefazolin (1 g, intravenous (IV)) was administered pre-implantation, and Amoxicillin (500 mg/day) was given one day before surgery and continued for 10 days post-implantation. The canines were pre-anesthetized with Pentothal Thiopental Sodium 2% at a dose of 25 mg/kg and maintained at the surgical plane with Halothane.

The first step was to harvest the autologous canine jugular vein ECs. For this evaluation, 6–10 cm segments of both jugular veins were

excised. Each excised segment of the jugular vein was placed in sterile Medium-199 (Gibco Life Technologies) for transport to a sterile laminar flow tissue culture hood. The excised vein segments were then inverted over a 5.0 mm diameter, sterile glass rod, exposing the luminal EC-lined surfaces. The everted veins were then placed in a 0.2% solution of collagenase (Type I, Sigma Chemical Co.) in DPBS for 20 min at room temperature [14,15,18]. The ECs were irrigated from the vein surfaces with DPBS. The EC–DPBS suspension was then centrifuged (110 g, 6 min), rinsed, recentrifuged, and resuspended in the seeding DPBS (5×10^6 EC/mL).

Graft EC Seeding

The grafts (length = 6 cm) were seeded with the harvested canine jugular vein ECs by the standard electrostatic seeding technique using the determined applied voltage/seeding time (+ 2.0 V/16 min). Again, the applied voltage for the CardioTech graft was determined by the previously described *in vitro* pilot study. After electrostatic EC seeding, the graft was placed in fresh, sterile Medium-199 (containing a Penicillin–Streptomycin–Fungizone® mixture (Whittaker M.A. Bioproducts, Inc.; 1.25% (vol.)), composed of 10,000 Units penicillin/mL, 10,000 Units streptomycin/mL, and 25 UG amphotericin-B/mL) and transported back to the surgical unit for implantation. The total time, from vein harvesting to completion of seeding was approximately 70 min.

Bilateral Canine Implantation

The control graft (non-EC seeded) was implanted while the EC harvesting and seeding of the test graft was being completed. The implantation of the bilateral, femoral grafts was randomized. A 10 cm length of each (left and right) femoral artery (FEM) was isolated bilaterally. The FEM site was chosen for this study because it closely simulates the conditions found in human lower extremity vasculature such as a FEM-popliteal and FEM-peroneal graft. Bypass procedures at these locations are prime candidates for the use of small-sized prostheses being investigated here. The conditions in the FEM include a vessel inner diameter of approximately 2–6 mm with flow rates comparable to those of humans, producing an average wall shear stress of 15 dynes/cm^2 [14]. Five minutes prior to cross clamping of the vessels, an IV heparin (50 U/kg) injection was administered. Each vessel was then cross-clamped and a 4–6 cm segment of the FEM removed. The grafts (both control and EC seeded) were then used to bypass

these excised sections using an end-to-end anastomosis with 7-0 suture. Immediately after implantation, each graft was palpated for a pulse to insure patency. A total of eight ($n = 8$) dogs were used in the preliminary six-week *in vivo* research protocol with an additional two canines used in a preliminary four-week study.

Graft Retrieval

After the four- or six-week implantation period, the grafts were harvested for detailed examinations. The grafts were excised following a standard procedure. The canines were anesthetized and maintained at the surgical plane with sodium pentobarbital while the graft segments were isolated bilaterally. Two silk sutures were wrapped around the proximal FEM, approximately 1 cm apart, to stop blood flow two centimeters upstream of the proximal anastomosis. Five minutes prior to any blood flow restriction with the vessels, an IV heparin (50 U/kg) injection was administered. A tapered one-way valve was then placed in the FEM through an incision between the two suture ties. A syringe filled with formalin was then attached to the one-way valve to flush and fill the grafts. A silk tie was used to close off the FEM two centimeters downstream of the distal anastomosis. The graft was then perfused with Formalin and resected from the canine. The grafts were excised by cutting the native FEM at each end without disturbing the graft. After at least 18 h in formalin, the graft segments were cut along the longitudinal midplane (proximal half only), and a visual examination of the luminal surface was conducted before it was subjected to the procedures below. After graft retrieval, the canine was euthanized via an IV injection of Euthasol following a standard protocol.

Necropsy Examination

Representative anastomotic arterial and graft specimens were sectioned to provide appropriate and adequate specimens of the proximal anastomosis (native artery and graft), mid-graft area, and the distal anastomosis (native artery and graft) of the grafts. Standard paraffin-embedding and sectioning procedures were followed for the preparation of glass slides for histological review. This procedure included dehydration with increasing concentrations of ethanol, infiltration with xylene, embedding in paraffin, and thin sectioning with a microtome. One slide per graft segment (3–4 segments per graft) was made, each containing 2–6 cross-sectional slices of the graft per slide. These specimens were then stained with Hematoxylin and

Eosin (H & E). This stain permits evaluation of the degree and extent of the healing process consisting of neointima formation, interstitial graft healing, and thrombosis.

The stained slides were subjectively evaluated by light microscopy for the degree of: (1) neointima (EC lining) development and (2) thrombosis. The histological examination was conducted by a trained, ''blinded'' individual on samples which were identified only by number with no reference to the origin of the samples. The magnitude of each of these parameters was ranked with a subjective scale as follows: 0 (absent), 1 (mild), 2 (moderate), and 3 (marked). A histological evaluation record was completed for each sample. The results from the histological examination are expected to provide more details on the acute healing/remodeling process. It is expected that the electrostatically EC seeded grafts will demonstrate a more complete neointimal development and reduced thrombus formation as compared to the controls.

RESULTS AND DISCUSSION

In Vitro Study

Prior to initiating the *in vivo* evaluation, a limited preliminary *in vitro* evaluation was performed. This study was to evaluate by SEM the effect of electrostatic EC seeding on the CardioTech vascular graft material. As previously noted, the 3 cm (length) graft segments were electrostatically seeded ($0.0, +1.0, +2.0,$ and $+5.0$ V applied to the prototype apparatus) for a constant 16 min seeding period (5×10^6 HUVEC/mL). The results of this study involved only one ($n = 1$) data point for each voltage/time combination evaluated. The SEM micrograph of the control, unseeded graft is presented in Figure 2A. Figure 2B (gravitational seeding, 0.0 V/16 min) illustrates that the ECs were seeded at a low seeding density, as well as low morphological maturation (i.e., the majority of the ECs were still spheroid morphology). The result of the $+1.0$ V/16 min seeding is shown in Figure 2C. Again, the results of this seeding combination resulted in low seeding density (greater than the 0.0 V seeding) along with low cell morphological maturation. The result of $+2.0$ V/16 min is illustrated in Figures 2D and 3. This seeding condition resulted in an excellent graft luminal surface coverage of ECs at very high morphological maturation (i.e., the ECs are completely flattened). The final time/voltage combination evaluated was $+5.0$ V/16 min (Figure 2E). At this seeding condition, the cells were being fused together. Upon rinsing the graft after seeding, a distinctive

gel (slim) was seen within the graft indicating cell fusion. This result is clearly evident in Figure 2E, with large clumps of fused cells seen lining the graft lumen. After this preliminary *in vitro* evaluation, the optimum electrostatic seeding for 4-mm I.D. CardioTech vascular grafts was

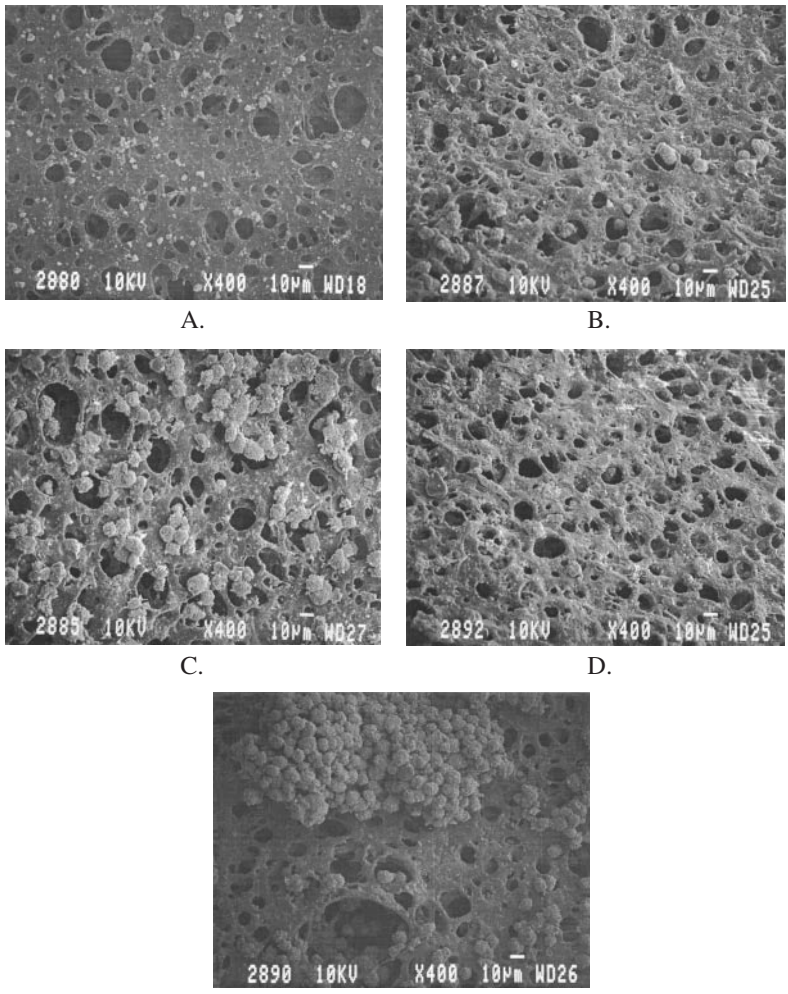


Figure 2. SEM micrographs of the CardioTech graft luminal surface after electrostatic EC seeding with HUVECs. A. Control graft that was not seeded. B. After gravitational seeding (0.0V applied to seeder). C. Electrostatically seeded at +1.0 V. D. Electrostatically seeded at +2.0 V. E. Electrostatically seeded at +5.0 V. All seeding times were held constant at 16 min, and all the micrographs are at 400X magnification.

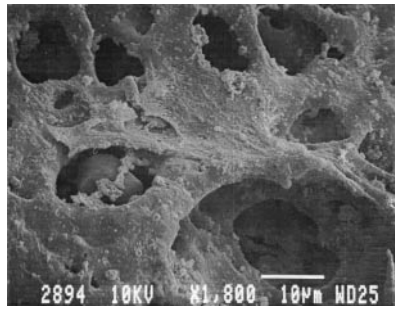


Figure 3. Higher magnification (1,800X) SEM micrograph of the CardioTech graft luminal surface electrostatically seeded with HUVECs at + 2.0 V for 16 min to demonstrate EC morphological maturation.

determined to be + 2.0 V/16 min. Thus, this seeding condition was used for the preliminary *in vivo* evaluation.

Another result from the *in vitro* evaluation was that the CardioTech vascular graft material adsorbed the EC suspension fluid; in some cases, it seemed that the graft wall adsorbed the entire fluid medium. The electrostatic seeding was continued utilizing this phenomenon. It is speculated by the authors that this fluid adsorption aided in the delivery of the ECs to the graft luminal surface, while the electrostatic seeding aided in accelerating morphological maturation. Thus, in all experiments, the seeding was performed on dry, out of the package, vascular graft segments to make use of both phenomena to enhance EC seeding.

In Vivo Results

The *in vivo* results will be reported in terms of the results for each canine in the order in which they were enrolled in the study. No statistical analysis of the histological data was performed due to the low number of animals in the four- and six-week studies. All data presented in this section, unless otherwise noted, is for the examination of the mid-graft regions only.

The first canine enrolled in the six-week implantation study had a Prothrombin time of 9.1 s. Upon explantation, neither of the grafts were patent. The problem encountered with this canine was that both proximal arteries appeared to have been stretched, which narrowed (necked) the lumen. In the case of the control graft, the proximal artery had a measured external diameter of 2 mm. For the seeded graft, the proximal artery was also necked down to 2 mm outer

diameter; thus, this necking compromised flow through the grafts. The distal vessel in each case was normal size (3–4 mm outer diameter). Visual examination of both grafts revealed a 1–1.5 cm long, cone-shaped (white in color), hyperplastic response grown into the proximal end of each graft. As for the SEM luminal surface evaluation, both grafts showed a light layer of thrombus, with even coverage. The control graft on top of the even coating had areas of large thrombus or islands on the surface. The histological evaluation of both mid-graft segments revealed mild to no thrombus formation and no signs of neointimal development.

For the second canine in the six-week study, the EC seeded and control grafts were patent as determined by the presence of a distal pulse, as measured at the distal femoral arteries. This canine had a Prothrombin time of 6.7 s. The seeded graft demonstrated perfect healing of all tissue and moderate to maximum graft encapsulation (difficult to excise graft – primarily at anastomoses). Visual examination of the EC seeded graft luminal surface (proximal end only) showed minimal thrombus coating the entire graft surface. The control graft demonstrated normal graft encapsulation. The site of incision had an open (1 cm) wound that did not seem to affect subcutaneous healing. This is normal and probably due to excessive chewing of the wound site by the canine. Visual examination of the control graft revealed that the entire graft was full of thrombus. Upon explanation of both grafts, a mature clot (the length of the graft segments) was flushed from the grafts during perfusion with formalin. Detailed SEM examination of the graft luminal surfaces indicated that the EC seeded graft did indeed have a section of neointima developed in the mid-graft region (Figure 4). The developed endothelium appeared mature and was aligned with the direction of flow through the graft. This neointima was delaminating but was seen by SEM and histology. The histological evaluation of both mid-graft segments revealed mild to moderate thrombosis within the EC seeded graft segment showing minimal signs of neointimal development (Figure 5). The control graft showed no signs of neointimal development (Figure 6). Another result from the histological review was that the neointimal development seen was only covering about one-fourth of the graft luminal surface. It is hard to tell exactly what effect the indwelling thrombus might have had on EC viability over the longer term, and it is, thus, very difficult to expand upon the results presented.

Canine number three (Prothrombin time: 8.5 s) in the six-week study had obviously been chewing at the wound sites on both legs approximately 1 cm below the external wound suture line. No open

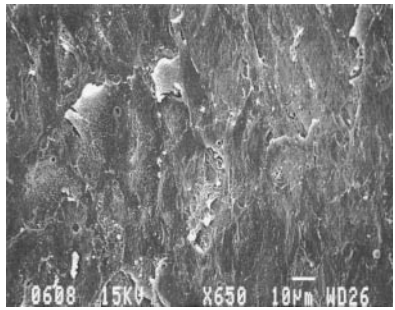


Figure 4. SEM micrograph of the electrostatically seeded CardioTech graft luminal surface implanted for six weeks in the canine femoral artery. *Note*: The exact direction of flow is unknown. However, the long axis of the implanted graft is aligned from top to bottom of this photo. Flow direction is, therefore, either from top to bottom or bottom to top.

wounds were visible, but bruising in parallel lines was apparent. The seeded and control grafts were both patent. The seeded graft exhibited minimal encapsulation. In fact, upon removal of the muscle layer from above the graft, the graft literally popped right out of the tissue. This graft also displayed essentially no scar tissue formation at the anastomoses, which was not seen in the majority of the cases. The seeded graft's subcutaneous tissue seemed to heal normally. No signs of infection were apparent in the seeded wound area. Visual examination of the seeded graft luminal surface showed no proximal hyperplasia but did show miscellaneous thrombus throughout the graft. The control graft was substantially more encapsulated and more difficult to isolate. At the anastomoses, it also had a large amount of scar tissue compared to the seeded graft. In the local wound area, the control graft's subcutaneous tissue healed very nicely, but there was some serous fluid present subcutaneous; this was probably brought about by chewing of the wound site. Visual examination revealed no proximal hyperplasia and no noticeable thrombus on the control graft luminal surface. The SEM examination revealed extensive thrombus formation on both graft luminal surfaces. Both grafts were full of thrombus. However, the thrombus layer was delaminating off of the seeded graft luminal surfaces but not from the control graft. The histological evaluation revealed moderate to maximum hyperplasia at the distal end of each graft. The mid-graft sections revealed no neointimal development with mild to moderate thrombus. There was also moderate to maximum hyperplasia at the distal ends of the grafts.

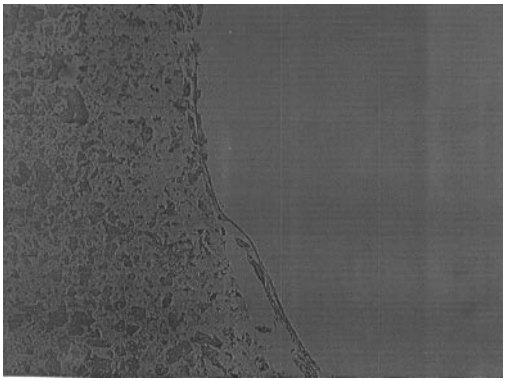


Figure 5. Histological section micrograph of the EC seeded CardioTech graft luminal surface implanted for six weeks in a canine femoral artery.

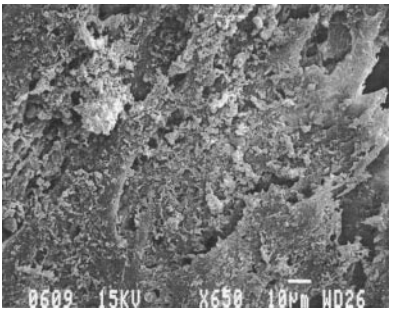


Figure 6. SEM micrograph of the control CardioTech graft luminal surface implanted for six weeks in a canine femoral artery.

The EC seeded and control grafts were patent as determined by the presence of a distal pulse (weak) in the distal femoral arteries for the fourth canine in the six-week study. The seeded graft demonstrated perfect healing of all tissue and normal graft encapsulation. The control graft demonstrated normal subcutaneous healing and graft encapsulation. In both cases, the grafts could not be cannulated for flushing due to what was determined later to be proximal hyperplasia and thrombosis. Visual examination of the seeded graft revealed a 1–1.5 cm long, cone-shaped (white in color), hyperplastic response grown into the proximal end of the graft. Visual examination of the control graft revealed that the entire graft was full of thrombus. This canine had a Prothrombin time of 6.5 s. The SEM evaluation of the seeded graft revealed that the graft luminal surface

was coated/covered by a layer of thrombus. The control graft was loaded with a thick layer of thrombus. Histologic evaluation revealed none to mild thrombus on the EC seeded and control mid-graft sections. The mid-graft section of the control revealed no neointimal development. The EC seeded graft showed what looked to be an EC lining or the remnants of an EC lining.

For canine number five in the six-week study, the EC seeded graft was patent, but the control graft was not as patent, as determined by the presence/absence of a distal pulse in the distal femoral arteries. The Prothrombin time for this canine was 6.9 s. The seeded graft demonstrated perfect healing of all tissue and mild graft encapsulation (easy to excise the graft). Visual examination of the EC seeded graft luminal surface (proximal end only) showed a layer of thrombus coating the entire graft surface. The control graft demonstrated minimal to no graft encapsulation with very poor healing of the tissue surrounding the graft; excessive chewing by the canine of the wound site probably caused this. Upon isolation of the graft, the distal anastomosis was partially disrupted (only a few sutures holding the graft in place). The proximal end of the control graft was occluded with clot (did not seem to be hyperplasia). It was obvious that the proximal end had been occluded prior to the distal anastomosis disruption; otherwise, the canine would have bled excessively. No bleeding or signs of bleeding were seen in the wound area. The SEM examination of the EC seeded graft luminal surface showed a thin layer of thrombus, much thinner than the graft wall thickness, with a large number of recently activated platelets visible. The control graft had a thicker, more developed coverage of thrombus, with a large thrombus area (island) seen on the luminal surface. This thrombus layer was somewhere between a few activated platelets on the surface and a completely occluding clot formation. The histological section results showed that there was no neointimal development on the graft luminal surfaces, with mild to moderate thrombosis seen on the control and seeded grafts, respectively. One note is that the control graft histology sections revealed a moderate degree of hyperplasia at the distal end but no proximal hyperplasia. The seeded graft histology sections of the proximal end of the graft revealed a mild/moderate degree of hyperplasia.

Upon explantation of the grafts from canine number six in the six-week study, the control graft was not patent but the seeded graft was still patent. For this canine, the Prothrombin time was 7.0 s. The control graft demonstrated perfect healing of all tissue and normal graft encapsulation. The seeded graft had a small open wound in the middle of the closure wound. The seeded graft also had a small ''knot'' of

tissue that showed no signs of infection. All other subcutaneous healing on the seeded side, as well as graft encapsulation, was normal. Visual examination of the seeded and control grafts revealed thrombus uniformly covering the luminal surface. SEM examination of the seeded and control grafts revealed a thin layer of thrombus uniformly covering the luminal surface with small islands of thrombus visible on top of the luminal coverage. The histological evaluation revealed none to mild thrombus on the EC seeded and control graft mid-sections. The mid-graft sections of both grafts revealed no neointimal development.

No data is available for canine number seven in the six-week study, as it was never revived from anesthesia. An autopsy revealed stringy mature thrombus within the right ventricle. No signs of heart worms were apparent but there may have been remnants from treatment of heartworms. The left lung was also collapsed.

The final canine enrolled in the six-week study had a Prothrombin time of 10.5 s. Upon explantation, the control and EC seeded grafts were not patent. Both the control and seeded grafts demonstrated perfect healing of all tissue and normal graft encapsulation. Visual examination of the seeded and control grafts revealed thrombus uniformly covering the luminal surfaces. The SEM analysis of the seeded graft luminal surface revealed a light and uniform layer of thrombus covering the entire graft. In some regions, the thrombus was absent, and the graft material was visible. The control graft was loaded with a thick layer of thrombus, and many larger islands of thrombus covered the graft luminal surface. The histological evaluation revealed mild to moderate thrombus on the EC seeded and control graft mid-graft sections. The mid-graft sections of both grafts revealed no neointimal development.

In summary, the results from the first eight canines enrolled in the study were very limited as to the desired results of graft patency and endothelialization after the six-week implantation period. This seems to be primarily due to an abundance of proximal anastomotic hyperplasia. After reviewing these results, several modifications were made to the protocol, and two additional canines were enrolled in the study for further evaluation of the electrostatic EC seeding of CardioTech 4-mm I.D. vascular grafts. Because of this, no detailed results including statistical analysis is presented.

Additional Canine Studies Performed (Four-Week Implants)

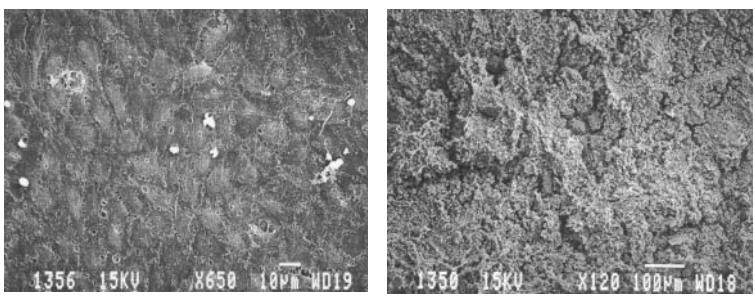
The modifications to the original six-week study protocol are as follows. First, larger canines ($n = 2$) (32–33 kg; male) were used in this study in an attempt to get more appropriate size matching between

the femoral artery and the 4-mm CardioTech vascular graft. The second addition to the protocol was soaking the grafts in a heparin solution prior to implantation. For this soak, the control grafts were placed in the graft transport medium supplemented with 50 Units/mL of heparin (Sigma Chemical Co.). The control grafts were allowed to soak in this medium for a minimum of 30 min. The EC seeded grafts were also soaked in the heparin-supplemented transport medium after the seeding procedure was complete for a minimum of 30 min. The final protocol adjustment was the implantation period. Due to time constraints, the grafts were implanted for a four-week period instead of the original six weeks. The remainder of the protocol is identical to that described for the first eight canines.

The first of the canines enrolled in the four-week study had a Prothrombin time of 7.0 s. Upon explantation, the EC seeded graft (faint distal pulse) was patent while the control graft was not, as determined by the presence/absence of a distal pulse in the distal femoral arteries. The control graft had a 2 cm long open wound, which was draining. The subcutaneous tissue was ''mushy,'' which rendered minimal to no graft encapsulation. No signs of infection (no pus seen in the wound) were apparent. Visual examination of the control graft revealed a 1 cm long, cone-shaped (white in color), hyperplastic response grown into the proximal end of each graft. The seeded graft demonstrated good healing of all tissue with mild graft encapsulation (easy to excise the graft). Visual examination of the EC seeded graft luminal surface (proximal end only) showed a fairly thick layer of thrombus coating the entire graft surface. The SEM analysis of the seeded and control graft luminal surfaces revealed a light, uniform layer of thrombus covering the entire graft. In some areas, the thrombus was absent, revealing the graft material. The histological evaluation exhibited mild to moderate thrombus on the EC seeded and control graft mid-graft sections. The mid-graft sections of both grafts revealed no neointimal development.

Upon explantation of the grafts from the second canine in the four-week study, both grafts were patent as determined by distal pulses. Both external suture lines had small (<1 cm long) surface wounds and small bumps (swelling) at the mid-suture lines. The seeded graft side had a strong distal pulse along with good subcutaneous healing and normal graft encapsulation. No signs of infection were present on the seeded graft side. The visual examination of the proximal half of the graft revealed a clean, pristine luminal surface. No thrombus was seen at the proximal end (half) of the graft. This canine had a Prothrombin time of 7.0 s. The control graft external wound had a substantial amount of pus throughout the wound site. Due to this

pus (infection), the control graft was not encapsulated at all. Visual examination of the control graft luminal surface revealed a small amount of pannus ingrowth (EC migration from native artery), with the distal artery occluded by a thrombus formation. In this case, the clot seems to have been dislodged from within the graft during the perfusions with formalin. Detailed SEM examination of the graft luminal surfaces indicated that the EC seeded graft did indeed have an immature neointima developed in the mid-graft region (Figure 7A). The developed endothelium looked immature and partially aligned with the direction of flow through the graft. When referring to immature neointima, the authors mean that the ECs seen were more rounded than elongated in the flow direction. The ECs also had what appeared to be pseudopodia extending from a majority of the cells. Thus, it is the opinion of the authors that the neointima is immature but present. This neointima was not delaminating but seemed to be nicely adhered to the graft luminal surface (Figures 7B and 8). It should also be noted that an island of thrombus was seen on the seeded graft luminal surface at 90° to the section with neointimal development. The control graft luminal surface had a thick layer of thrombus on the graft luminal surface similar to that seen in Figure 7B except for a more expansive coverage. The histological review reiterates the results seen on the SEM. The histological evaluation revealed none to mild thrombus on the EC seeded graft, with the control graft possessing mild thrombus formation. The mid-graft section of the seeded graft showed moderate neointimal development, with the control graft having no neointimal development.



A. B.

Figure 7. SEM micrographs of the electrostatically EC seeded CardioTech graft luminal surface implanted for four weeks in the canine femoral artery. A. This micrograph illustrates the immature neointimal development. B. This micrograph illustrates the small area, ''island,'' of thrombus that was seen on the electrostatically EC seeded graft and is also representative of the control graft luminal surface.

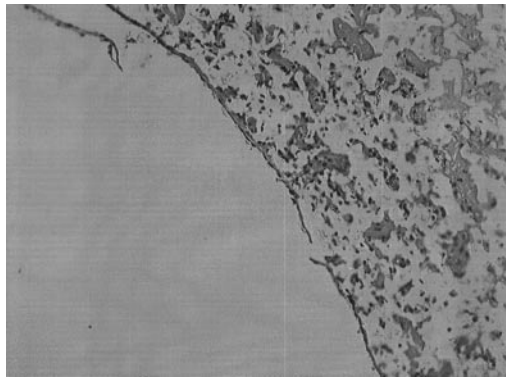


Figure 8. Histological section micrograph of the electrostatically seeded CardioTech graft luminal surface implanted for four weeks in a canine femoral artery.

Historically, the patency rates of small diameter synthetic vascular prostheses fail to match those of autologous vessel grafts when used clinically [19]. As previously discussed, the concept of electrostatic EC seeding was developed to overcome the limitations of past attempts at a clinically relevant EC seeding technique as a potential treatment to reduce the rate of failure of small diameter vascular prostheses. The ultimate goal of EC seeded small diameter vascular prostheses with long-term patency has not been achieved due to technical issues that include poor cellular adhesion to graft material and subsequent lack of retention upon implantation of the seeded grafts. As the previous and present *in vitro* and *in vivo* results have demonstrated, electrostatic EC seeding provides a means of overcoming both of these limitations [4–7,14]. Electrostatically enhanced EC attachment and accelerated morphological maturation (flattened ECs) [7] resulted in no significant EC loss upon physiologic shear stress exposure (*in vitro*) for up to 120 min, a time at which the bulk of the EC loss would have been expected [2]. It is speculated by the authors that this increased cellular retention may also reduce the need for large numbers of seeded ECs initially. Combining the canine *in vivo* results presented in this paper with those results previously obtained *in vitro* and *in vivo* [4–7,14], the electrostatic EC seeding technique shows promise in terms of a clinically acceptable protocol which could provide the necessary conditions to improve long-term patency rates of small caliber vascular grafts. Additional long-term (>18 weeks) *in vivo* animal data and subsequent clinical trials would be required to completely discern the vascular graft patency and the hyperplastic response associated with small diameter graft implantation.

One clinical concern is the time required to perform the EC harvesting and seeding procedures. For EC seeding to be routinely employed by clinicians, a minimal amount of time must be used to obtain maximum luminal coverage of seeded ECs. There is considerable debate over a clinically acceptable seeding time, with 2 h or less appearing to be a consensus. A time greater than 2 h would not be acceptable because of the possibility of genotype and/or phenotype changes from cellular exposure to an undefined media-containing serum [19]. Based on the experience of this study, the combination of EC harvesting and electrostatic seeding would take approximately 60 min (microvascular ECs), thus falling well within the clinically acceptable time frame.

CONCLUSION

The *in vitro* results indicate that $+2.0$ V for 16 min is the optimum electrostatic seeding condition for 4-mm I.D. CardioTech vascular grafts. Again, this is based on very limited data ($n = 1$) but, due to experience with e-PTFE grafts, it is most likely accurate. This was considered optimum because it led to high morphological maturation of the seeded ECs, while allowing for an efficient luminal surface coverage, yet without causing any obvious cellular damage. The results from the preliminary *in vivo* evaluation demonstrated that the electrostatically seeded ECs remained and were able to develop a neointimal lining on the CardioTech 4-mm vascular grafts implanted in a canine femoral artery.

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