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Flow Preservation of Umbilical Vein for Autologous Shunt and Cardiovascular Reconstruction

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Abstract

Background.—Synthetic graft materials are commonly used for shunts and cardiovascular reconstruction in neonates, but are prone to thrombosis and scarring. The umbilical vein is a potential source of autologous, endothelialized tissue for neonatal shunts and tissue reconstruction, but requires preservation before implantation.

Methods.—Umbilical cords were collected in UW solution with antibiotics at 4°C until dissection. Umbilical vein segments were tested for burst pressure before and after 2 weeks of preservation. Umbilical veins segments were preserved under static or flow conditions at 4°C in UW solution with 5% human plasma lysate for 7 days. Veins were evaluated with histopathology, scanning electron microscopy, and platelet adhesion testing.

Results.—Umbilical veins have no difference in burst pressure at harvest (n = 16) compared with 2 weeks of preservation (n = 11; 431 ± 229 versus 438 ± 244 mm Hg). After 1 week, static and flow-preserved veins showed viability of the vessel segments with endothelium staining positive for CD31, von Willebrand factor, and endothelial nitric oxide synthase. Scanning electron microscopy demonstrated preservation of normal endothelial morphology and flow alignment in the flow-preserved samples compared with cobblestone endothelial appearance and some endothelial cell loss in the static samples. Static samples had significantly more platelet adhesion than flow-preserved samples did.

Conclusions.—Umbilical veins have adequate burst strength to function at neonatal systemic pressures. Preservation under flow conditions demonstrated normal endothelial and overall

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vascular morphology with less platelet adhesion compared with static samples. Preserved autologous umbilical veins are potential source for endothelialized shunts or cardiovascular repair tissue for neonates.

The options for cardiovascular shunts, conduits, and patches are synthetic or tissue-based materials such as polytetrafluoroethylene (PTFE), autologous pericardium, homografts, or xenograft pericardium. In particular, neonatal shunts are typically created using PTFE grafts. Due to lack of endothelium, there is risk of acute thrombosis and late stenosis of these grafts. In part due to thrombosis, the in-hospital mortality of patients with a modified Blalock-Taussig (BT) shunt varies from 4% to 15%, with a morbidity rate of 8% to 13% including the need for extracorporeal membrane oxygenation support or shunt revision [1–4]. In addition to the risk for acute thrombosis, stenosis can also develop within the BT shunt, leading to reduced pulmonary blood flow and the need for reintervention or early reoperation. The development of stenosis in BT shunts is significant since at the time of shunt takedown, 64% of BT shunts have greater than 25% narrowing and 21% have greater than 50% narrowing [5].

In an attempt to reduce infection and thrombotic complications, several different BT shunt conduits have been attempted. These have included fresh and cryopreserved human saphenous vein grafts [6–8], bovine internal mammary artery grafts [9], and allogenic glutaraldehyde treated umbilical vein (UV) grafts [10]. Overall, the preliminary outcomes with these human allografts or xeno-grafts have been favorable in small series. Despite the success of these alternate conduits in small series, the PTFE graft remains the BT shunt conduit of choice.

In an effort to reduce the early and late failures of current BT shunts, we postulate that an autologous endothelialized conduit may minimize the risk of early thrombosis and late stenosis formation. A unique source of autologous endothelialized conduit tissue is the UV, harvested from the umbilical cord at the time of delivery. With over 59% to 68% of neonates with congenital heart disease and up to 97% of patients with hypoplastic left heart syndrome identified with prenatal ultrasound [11, 12], collection of the umbilical cord at birth seems feasible. However, the UVs would need to be preserved for at least several days, as most operative procedures for BT shunt or cardiovascular reconstruction are performed after a period of stabilization and imaging evaluation. Most of these children undergo an operation within the first week of life.

Integrity of endothelium is key component of tissue preservation that is relevant to materials used for BT shunt or cardiovascular repair. Endothelium provides a nonthrombogenic surface by covering the basement membrane and modulating both the coagulation pathways and platelet behavior at the vascular interface. In addition, endothelium serves an important role in modulating intimal and medial hyperplasia, which leads to conduit stenosis and tissue scarring. This study was therefore performed to evaluate cold preservation techniques for endothelium preservation. We hypothesize that preservation under flow conditions yields superior endothelial function compared with preservation under static conditions.

Material and Methods

Unless otherwise noted, all supplies were purchased from Sigma-Aldrich (St. Louis, MO).

Collection of Umbilical Cord Sample

Umbilical cords were collected at the Washington University of St. Louis, then subsequently at Brigham and Women's Hospital under institutional research board approval, and preservation and testing at Boston Children's Hospital under separate institutional research board approval. Umbilical cords were collected at the time of delivery from neonates without known congenital heart disease in a sterile container of cold University of Wisconsin (UW) solution (Organ Recovery Systems, Itasca, IL) plus penicillin, gentamicin, and amphotericin B (Life Technologies, Carlsbad, CA; or Zen Bio, Research Triangle Park, NC). Cords were preserved at 4°C until dissection of the UV between 1 and 24 hours after delivery. UVs collected for the final verification study were dissected immediately after delivery.

Dissection of Umbilical Cord

The UV was dissected from the umbilical cord using a sterile setup prepared within a dissection hood with care to keep the vein hydrated and to minimize dissection time. The UVs were then preserved under static or flow conditions. The umbilical cord internal diameter was measured in separate fresh segments using Hegar dilators at 1-mm increments. For the fresh samples collected immediately following dissection of the vein from the cord, 1-cm segments of veins were fixed after dissection: in optimal cutting temperature (OCT) freezing compound, or in 10% formalin or 4% paraformaldehyde (PFA). The OCT samples were stored at −80°C. The 10% formalin and 4% PFA samples were left at room temperature.

Static Preservation

Initially, veins were preserved under static conditions in 2% fetal bovine serum–containing endothelial cell media (EGM-2MV, Lonza, Allendale, NJ; n = 11) for 13 to 26 days in a standard 37°C incubator. These early samples demonstrated incomplete endothelial and vascular wall preservation. To improve preservation and adopt a transplant tissue approach, the preservation strategy was changed to cold preservation. UV segments were preserved under static conditions on a shaker table at 4°C at low speed in UW solution (n = 32 from 8 veins) or UW solution plus 5% human plasma lysate (HPL) (Cook Regentec, Indianapolis, IN) with penicillin, gentamicin, and amphotericin (n = 83 from 24 veins) at a ratio of 100 mL of media per centimeter of vein. A 0.22-mm filter was placed onto the lid to promote ventilation. The veins were preserved under these static conditions for 7 days. Static veins ranged from 0.5 cm to 4 cm in length. The segments were analyzed at the middle portions by histology, scanning electron microscopy (SEM), and platelet adhesion testing.

Bioreactor Flow Preservation

A custom bioreactor was developed to test flow preservation of the veins that housed up to 4 segments of 5 cm length veins. The bioreactor (Figs 1A, 1B) utilized a roller pump (MasterFlex, Gelsenkirchen, Germany) to generate flow within the veins. Additionally, a

commercially available bioreactor was utilized for flow preservation in the latter part of the study. The Lifeport Kidney Transporter system (Organ Recovery Systems) (Figs 1C, 1D) is a Food and Drug Administration–approved temperature- and pressure-controlled organ perfusion system in which the organ resides in a bath of perfusion solution maintained at 2° to 6°C. Although designed for kidney perfusion, the cannulation system was adapted for preservation of UV samples. Perfusion pressures were set at 2 to 12 mm Hg and a flow rate of 50 to 150 mL/min corresponding to 1 to 5 dynes/cm^2 shear stress, which is within the low normal range of venous shear stress. All flow-preserved samples (n = 56 segments, n = 21 veins) were preserved at 4°C using 750 to 1,000 mL of UW with 5% HPL for 7 days.

Histolopathological Evaluation

At the conclusion of the preservation under either static or flow conditions, 1-cm segments were frozen in OCT compound, sectioned at 7 μm and then stained with hematoxylin and eosin (H&E) for overall morphology, Movat pentachrome staining for extracellular matrix components, and immunohistochemistry for endothelium, including von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). Adjacent 1-cm segments were fixed in 10% formalin or 4% PFA and embedded in paraffin and sectioned for additional histopathology. Utilizing paraffin sections, fresh samples (n = 9 veins), static preserved samples (n = 14 veins), and flow-preserved samples (n = 18 veins) were analyzed by staining with H&E, Movat, and CD31.

Platelet Adhesion Testing

A platelet adhesion test was developed to evaluate the adhesion of platelets to the preserved UV segments. Sheep blood Na heparin (Lampire Biological Laboratories, Pipersville, PA) was kept in the refrigerator for up to a day before use. The blood was warmed up to 37°C and quinacrine dihydrochloride (mepacrine) was added(0.0047 g/L) to label the platelets. Mepacrine is a fluorescent dye that does not affect platelet function [13–15]. Fresh (n = 2 veins), static (n = 3 veins), and bioreactor (n = 7 veins) veins were tested as well as control silicone tubes with 3-mm × 5-mm-thin bovine collagen sheets in an exposed window as controls (DSM Biomedical, Exton, PA; n = 5 tubes). Static samples and the control silicone tubes with collagen sheets were inserted into couplings in the bioreactor before testing. The bioreactor was warmed up to 37°C, mepacrine was mixed in the blood for 30 minutes, and blood was pumped through the veins and collagen grafts for 30 minutes at flows of 150 mL•min•vein that corresponded to ~5 dynes/cm^2 shear stress. Upon completion, the veins were drained and rinsed gently with saline, cut open longitudinally, and placed in a dish with a small amount of saline. The veins were imaged under fluorescence with 488 nm filter using Axio Observer Z1 inverted microscope (Carl Zeiss, Jena, Germany). Platelet counts were obtained by averaging platelet numbers over 6 high-power fields.

Scanning Electron Microscopy

Further analysis of the endothelial cell confluence and morphology was performed by SEM. Fresh (n =4 veins), static (n = 5 veins), and flow-preserved (n = 8) samples were fixed in glutaraldehyde, paraformaldehyde and sucrose in sodium cacodylate buffer with postfixation in Palade’s osmium followed by dehydration with graded series of ethanol and then critical

point dried (Autosamdri-815, Tousimus, Rockville, MD). The samples were sputter coated with gold/palladium (Hummer 6.2 sputter coater, Anatech, Union City, CA) and imaged on a JEOL 5600LV scanning electron microscope (JEOL USA, Peabody, MA).

Verification Study

To evaluate the preservation methods in a simulated clinical situation, a verification study (n = 6 veins) was performed to confirm the preservation of the endothelium and media and to formally evaluate the sterility of the samples. Clinically sourced penicillin (WG Critical Care, Paramus, NJ), gentamicin (Hospira, Inc, Lake Forest, IL), and amphoteracin (X Gen Pharmaceuticals Inc, Big Flats, NY), as well as UW solution (Organ Recovery Systems) and human platelet lysate (Compass Biomedical, Hopkinton, MA), were used in the study. Umbilical cords were collected at birth following cesarean section delivery and stored in UW solution at 4°C. The UV was dissected from the cord in sterile fashion in a room adjacent to the delivery room within 30 minutes of birth. The UV was placed in the Lifeport device with preservation solution consisting of UW with 5% HPL, penicillin (102,000 μ/L), gentamicin (10 mg/L) and amphotericin (250 μg/L). Samples of the preservation solution were collected immediately after the vein was placed into the Lifeport device and sent for formal microbiology testing (Boston Children's Hospital clinical microbiology lab) for aerobic, anaerobic, and fungal cultures. The UVs were then preserved in the Lifeport device for 7 days at 4°C at pressures of 10 to 12 mm Hg and flow rates of 45 ± 8.8 mL/min (range, 31 to 63 mL/min). At day 7, a sample of the preservation solution was again sent for formal microbiology testing for aerobic, anaerobic, and fungal culture. Samples of the preservation solution (n = 3) were also sent for mycoplasma testing (WuXi AppTec, Philadelphia, PA). Samples of the UV were preserved in 4% PFA and evaluated at the time of dissection and after 7 days of preservation by staining for H&E, Masson's trichrome, α-smooth muscle actin, and CD31.

Statistical Methods

Values of vein diameter and burst pressure are expressed as mean ± SD. The mean counts of the platelet adhesion test were compared by Student's *t* test. A *p* value of <0.05 was considered to be statistically significant.

Results

Mechanical Properties

The diameter of the UVs varied from 4 to 7 mm, with a mean of 5.6 ± 0.83 mm (n = 21). UVs demonstrated no difference in burst pressure freshly after harvest (n = 16) compared with preservation for 2 weeks (n = 11; 431 ± 229 mm Hg versus 438 ± 244 mm Hg).

Histology and Immunohistochemistry Evaluation

Preservation at 4°C under static conditions for 7 days with UW without HPL resulted in evidence of cell necrosis in the media by H&E staining (data not shown). For static and flow-preserved veins in UW with HPL for 7 days, the vascular structure assessed by H&E and Movat staining is shown in Figure 2. Overall tissue shows preserved morphology and cellularity with improved overall vascular wall morphology in the flow-preserved

specimens. Specifically, in Figure 2, the static samples (Figs 2B, 2E) have a little more cuboidal shaped cells in the media with a more space between the cells, suggesting more edema and some loss of native cell alignment compared with the static (Figs 2A, 2D) and flow-preserved (Figs 2C, 2F) samples. Movat's staining demonstrates well-preserved extracellular matrix, including collagen (yellow), glycosaminoglycans (green-blue), and the internal elastic lamina in both the static and flow-preserved samples. The internal elastic lamina in the flow sample (Fig 2I) was more pronounced and appeared better preserved than the static sample (Fig 2H).

The integrity and functional properties of the endothelium was evaluated by several specific endothelium stains in the fresh and preserved samples. Figures 3 and 4 show the vWF, CD31, and eNOS staining. The vWF was relatively uniformly present for the fresh, static, and flow-preserved samples. The CD31 (Fig 3) was confluent in the fresh and flow-preserved samples, and present but not completely confluent in the static samples. As all of the veins were cultured in human platelet lysate, vWF in the plasma might have contributed to the relative uniform staining of vWF in the static and flow-preserved samples. NOS was present but never confluent in the fresh samples. It was similar in the static and flow-preserved samples with less than half confluency in each (Fig 4). The ICAM-1 and VCAM-1 stains were negative for all static and flow-preserved samples, suggesting an absence of activation of the endothelial cells (Fig 4).

Platelet Adhesion Testing

Platelet adhesion testing was compared between control and veins preserved under various conditions. The control samples with collagen sheets had robust accumulation of platelets, whereas veins preserved under static or flow conditions had significantly less platelet adhesion (Fig 5). Similarly, there was significantly less platelet accumulation in veins preserved under flow conditions compared with static conditions.

SEM Analysis

Imaging of the fresh specimens demonstrated intact endothelium that was aligned in the direction of flow (Fig 6). The segments preserved under static conditions had cobblestone appearing endothelium that was not flow aligned and demonstrated patches of missing endothelium with exposed basement membrane. In contrast, veins preserved under flow conditions had phenotypically normal endothelium that was confluent and aligned in the direction of flow without patches of exposed basement membrane.

Verification Testing

There was no evidence of infection in any of the verification samples at the time of dissection or after 7 days of preservation (n = 6). The verification samples after 7 days of culture (n = 3) were negative for mycoplasma. By H&E and Masson's trichrome staining, the preserved samples demonstrated intact extracellular matrix similar to that shown in Figure 2. The preservation of the media was excellent as demonstrated by α-smooth muscle actin staining at dissection and 7 days of preservation (Fig 7). By CD31 staining, the endothelium appeared to be intact and qualitatively similar after 7 days of preservation to the veins at the time of dissection (Fig 7).

Comment

This study demonstrates techniques for preservation of UV harvested at the time of delivery as a source of autologous, endothelialized tissue material for neonatal shunts and cardiovascular reconstruction. The histology, SEM, and functional assessments performed in this study demonstrate preservation of endothelial integrity as well as vein mechanical strength. Preservation of vein segments under static versus flow conditions demonstrates superior endothelial alignment and integrity with the flow preservation.

Thrombosis, stenosis and scarring following implantation of prosthetic materials have prompted consideration of alternative autologous, endothelialized tissue substitutes for neonatal applications as a systemic to pulmonary artery shunt. PTFE grafts, used for BT shunts in neonates, are particularly prone to early thrombosis and stenosis formation, and contribute morbidity and mortality. There are several potential advantages to use of preserved UV as an autologous endothelialized conduit in these patients. First, UV tissue is readily available in long segments at delivery, and is otherwise typically discarded. Prenatal diagnosis allows identification of most appropriate patients and planning of tissue harvest and preservation at the time of delivery. The caliber of the UV (4 to 7 mm diameter) can be easily adapted for use as a neonatal BT shunt. In theory, intact, healthy endothelium would to be the most important single aspect of an ideal shunt conduit. The challenge in bringing this concept to the clinic is the preservation of the vessel, especially the endothelium, for the duration of time between birth and implantation. Thus, our initial effort has focused on identifying the preservation conditions that result in optimal endothelial preservation for at least 1 week.

Our strategy for cold preservation in UW solution at 4°C utilized was chosen based on previous experiences. Currently for liver transplantation, extra segments of iliac artery and vein are harvested and preserved in UW or a similar solution in a static container at 4°C, and may be utilized up to 2 weeks after transplant [16, 17]. Human plasma lysate was added to the UW solution based on previous data showing that that the growth factors contained by the platelets are an option for a clinical substitute for fetal bovine serum to support endothelial cell and mesenchymal stem cell growth [18–20].

Several different methods were utilized to assess adequacy of endothelium. The central histologic assessment of endothelial integrity included histopathological staining for CD31, vWF, and eNOS, which are well-known markers of endothelium. The use of human platelet lysate may have contributed to the uniform vWF staining in the samples. This may explain why the difference between the CD31 staining and vWF staining. CD31 therefore will be the more discriminating stain in this application. The absence of ICAM and VCAM staining suggested that the endothelial cells were not activated or injured during their time in preservation.

Platelet adhesion test was utilized to evaluate the function of the endothelium in preventing platelet adhesion, especially important if the vein is to be used as a BT shunt conduit. The segments preserved under flow conditions demonstrated less platelet adhesion compared those preserved under static conditions. This method of endothelial assessment has been

validated in previous studies [13–15] and is especially relevant for eventual neonatal BT shunt clinical application. The SEM analysis demonstrated an impact of preservation under flow versus static conditions. The samples preserved under static conditions had abnormal appearance compared with fresh segments or those preserved under flow conditions, consistent with previous findings that flow stimulates endothelial alignment and behavior. The results suggest that preservation under flow conditions leads to superior endothelial integrity and function compared with preservation under static conditions.

The logistics of cord collection have proven feasible for vaginal and cesarean section deliveries. It is expected that for clinical application, the dissection may be best performed in a clinical area near the delivery room or operating room of the cesarean section. The cord would immediately be placed in UW solution containing antibiotics, and dissection of the vein from the cord would performed, and the segment would be mounted on the sterile closed system for preservation in UW solution with 5% HPL under flow conditions. The system could possibly be stored in a clinical area near the operating room until it was to be used for the BT shunt procedure or other neonatal reconstruction. All of these steps were performed for the verification testing, which confirmed the preservation of the media and endothelium without evidence of infection. The verification study samples qualitatively had even more robust endothelial preservation that observed when the UV was stored in UW solution for up to 24 hours before dissection. This slight qualitative improvement of endothelial preservation with early dissection and flow preservation certainly is not a surprise, but further testing is required to understand the implications for this clinically. However, from these data it appears to be ideal to immediately dissect the vein and preserve it under flow conditions. Further studies will be needed to delineate the impact, if any in waiting hours to up to 24 hours on the eventual endothelial preservation days later. Although this study demonstrated sterility up to only 7 days, future studies are necessary to determine the safe limit of preservation, as many neonatal operations occur beyond 7 days of age.

The choice of flow rate for flow preservation was determined by estimation of physiologic shear stress and intraluminal pressure present in normal UVs with the goal to have adequate flow to maintain normal endothelial phenotype and flow alignment. Lower flow rates are likely to be associated with endothelial disruption and malalignment as noted in static specimens, whereas supraphysiologic flow rates are likely to damage endothelium through excessive shear. However, further investigation is necessary to determine the optimal flow conditions for endothelial preservation.

Clinical application of these UVs as a BT shunt will require constraining the diameter to 3.5 mm (typical range, 3 to 4 mm). An external stent placed around the central aspect of the vein can provide this constraint and a degree of adjustability. The stent could be dilated in the cath lab at a later time if desired to provide a longer clinical utility of the shunt. Other clinical applications include neonatal right ventricle to pulmonary artery conduits and patch material for neonatal pulmonary artery reconstruction.

Conclusion

UVs have adequate burst strength to function in neonatal arterial circulation. Preservation at 4°C demonstrated retention of endothelium and overall vascular wall morphology, with flow

preservation samples having qualitatively better morphology with lower platelet adhesion than static preservation samples. Autologous UVs may serve as endothelialized BT shunt or cardiovascular patch material in neonates with the potential to reduce thrombosis and scarring.

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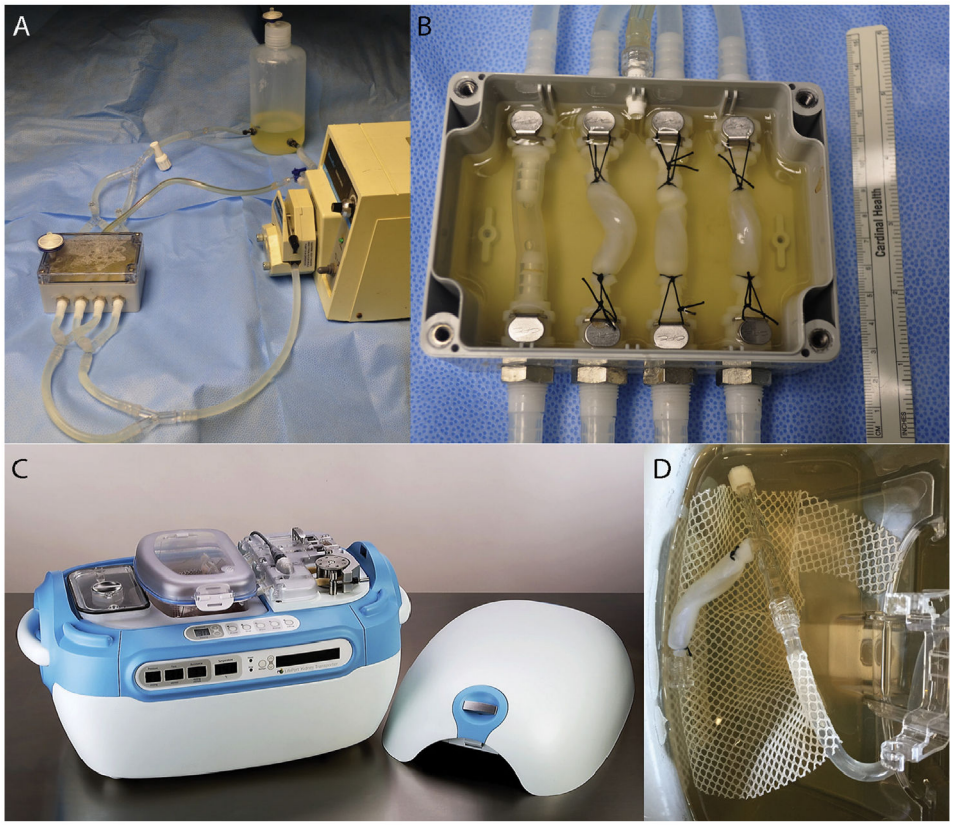

Fig 1.

(A, B) Bioreactor for flow preservation of 5-cm umbilical vein segments with 3 veins secured and preserved in flow conditions. (C, D) Lifeport transporter system with umbilical vein preserved under flow conditions within the Lifeport system.

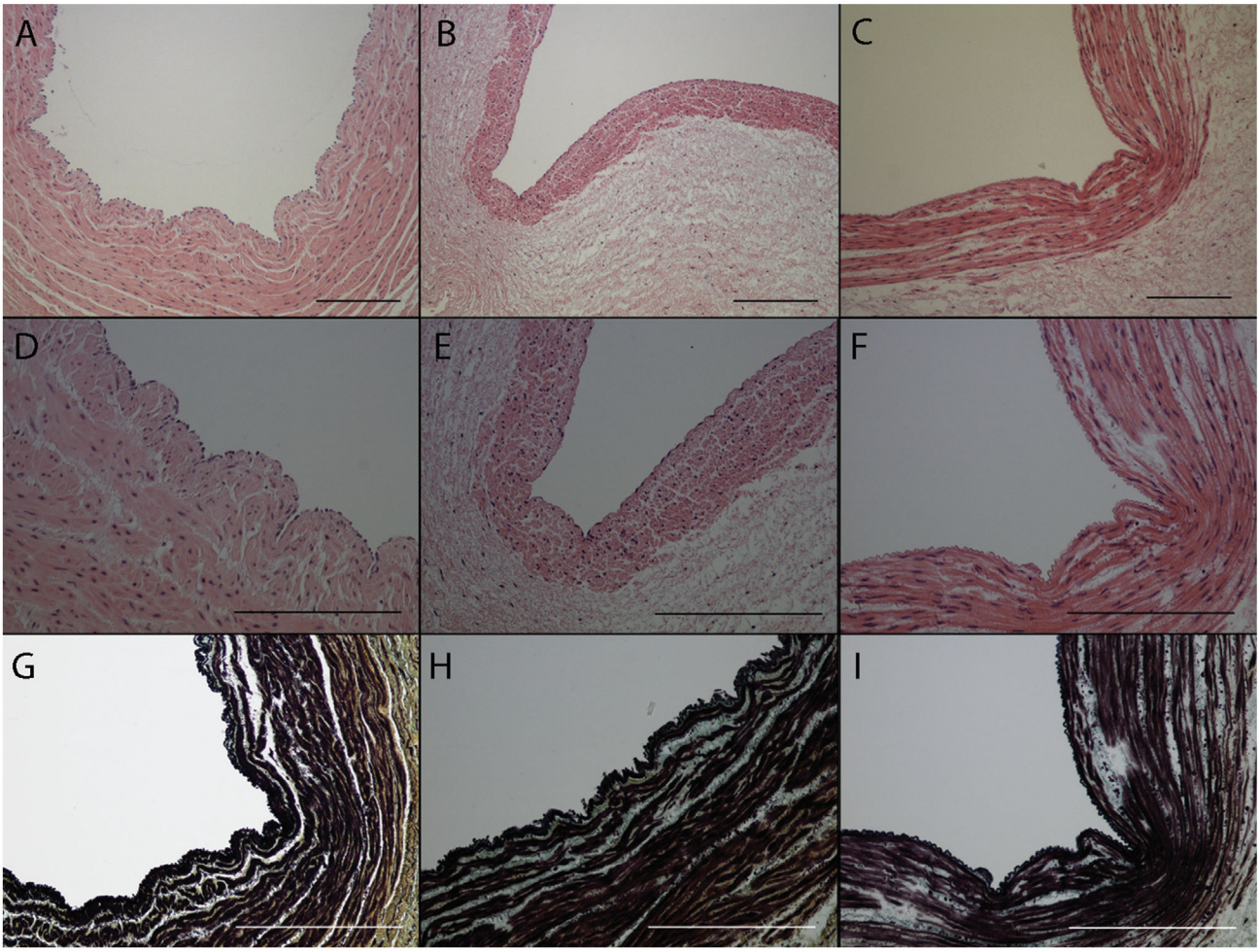

Fig 2.

Hematoxylin and eosin staining of paraffin samples of (A, D) fresh samples, (B, E) static preservation at 7 days, and (C, F) flow preservation at 7 days. Movat's staining of paraffin samples of (G) fresh, (H) static preservation at 7 days, and (I) flow preservation at 7 days. Scale bar = 250 μm.

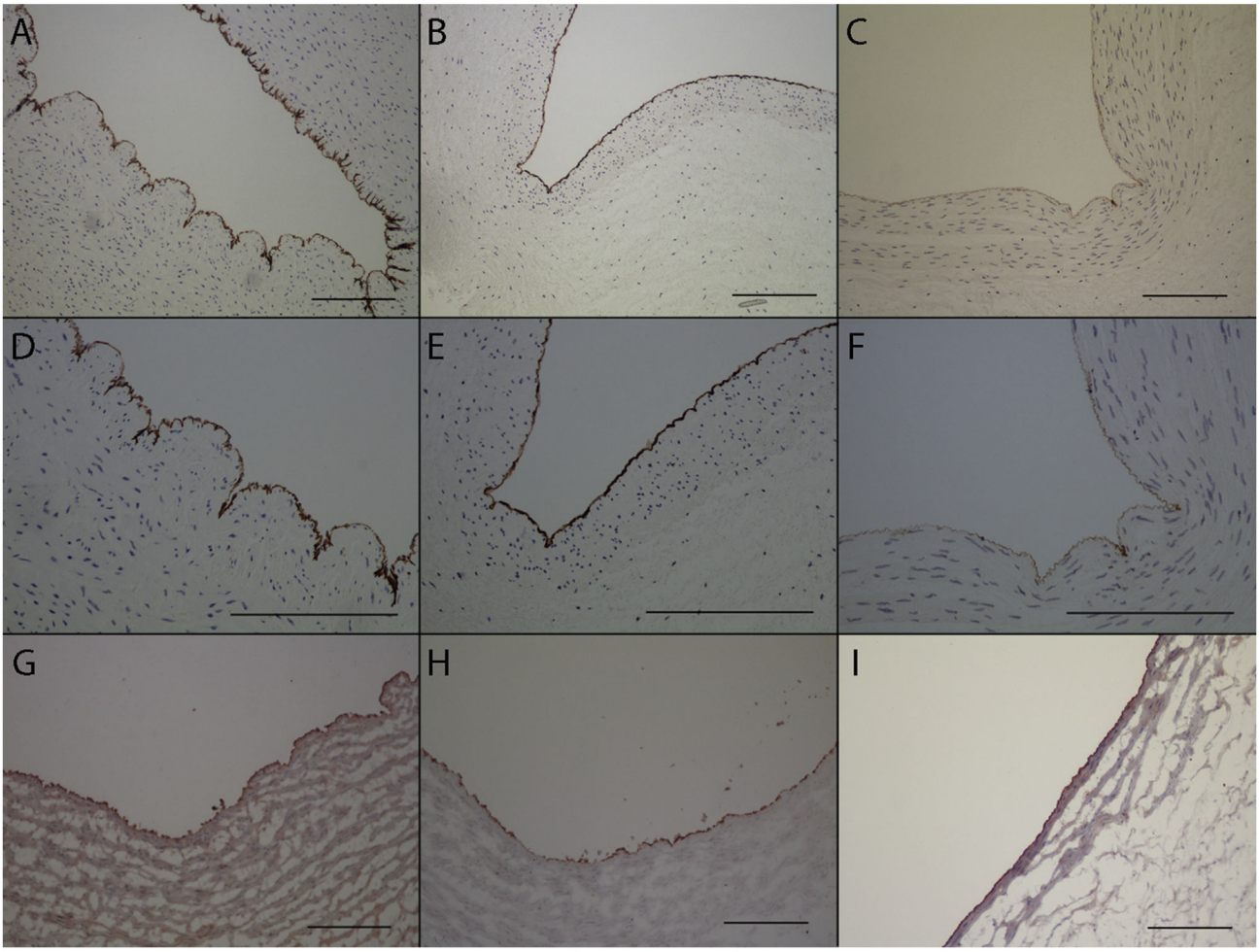

Fig 3.

CD31 staining of paraffin samples of (A, D) fresh samples, (B, E) static preservation at 7 days, and (C, F) flow preservation at 7 days. vWF staining of frozen samples of (G) fresh, (H) static preservation at 7 days, and (I) flow preservation at 7 days. Scale bar = 250 μm.

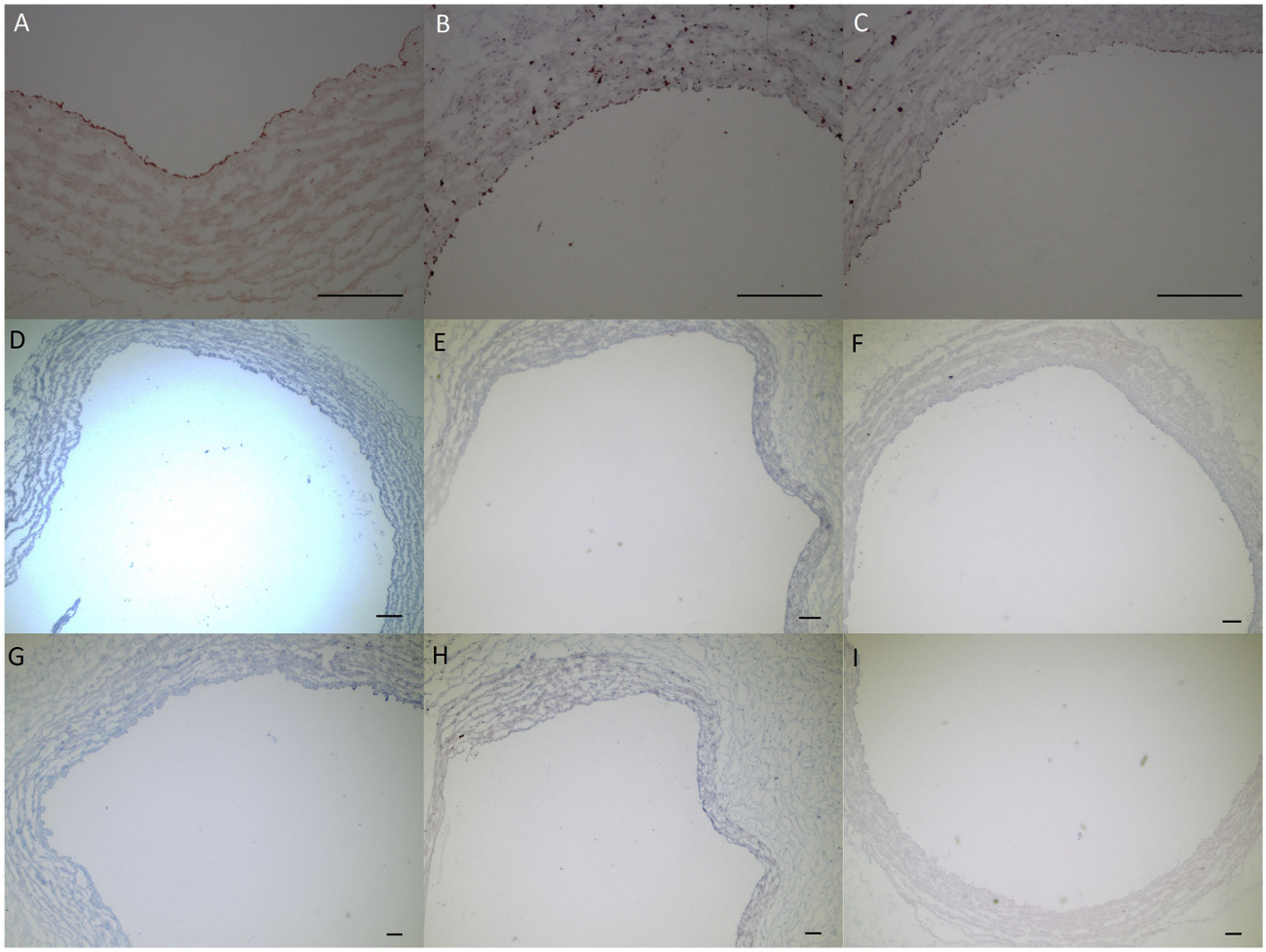

Fig 4.

Endothelial NOS staining of frozen samples of (A) fresh, (B) static preservation at 7 days, and (C) flow preservation at 7 days. Intercellular adhesion molecule staining of frozen samples of (D) fresh, (E) static preservation at 7 days, and (F) flow preservation at 7 days. Vascular cell adhesion molecule staining of frozen samples of (G) fresh, (H) static preservation at 7 days, and (I) flow preservation at 7 days. Scale bar = 250 μm.

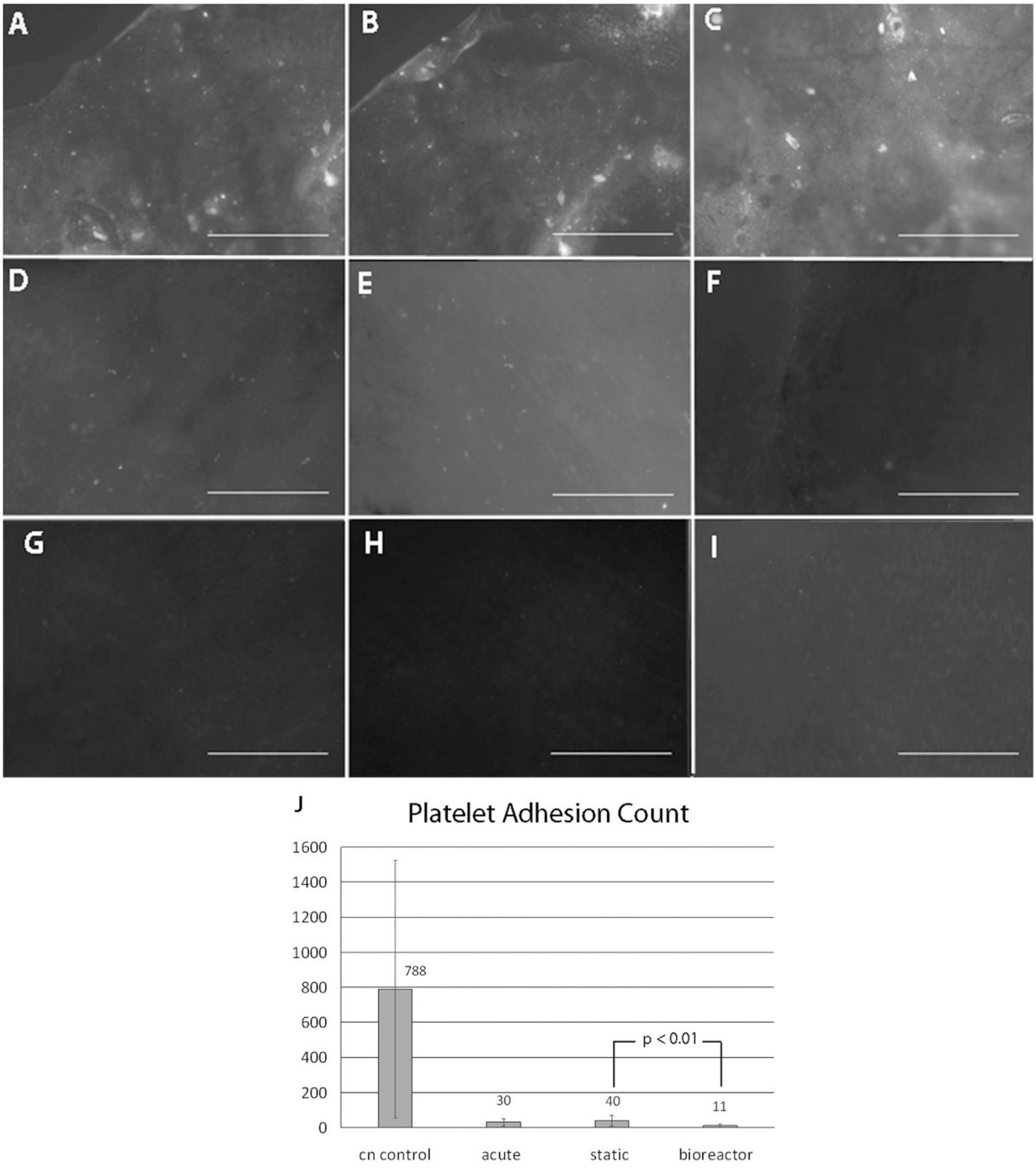

Fig 5.

Platelet adhesion test results of (A–C) collagen control samples, (D–F) static preservation 7-day samples, and (G–I) flow preservation 7-day samples, and (J) graph of counts of platelet adhesion testing. Scale bar = 500 μm.

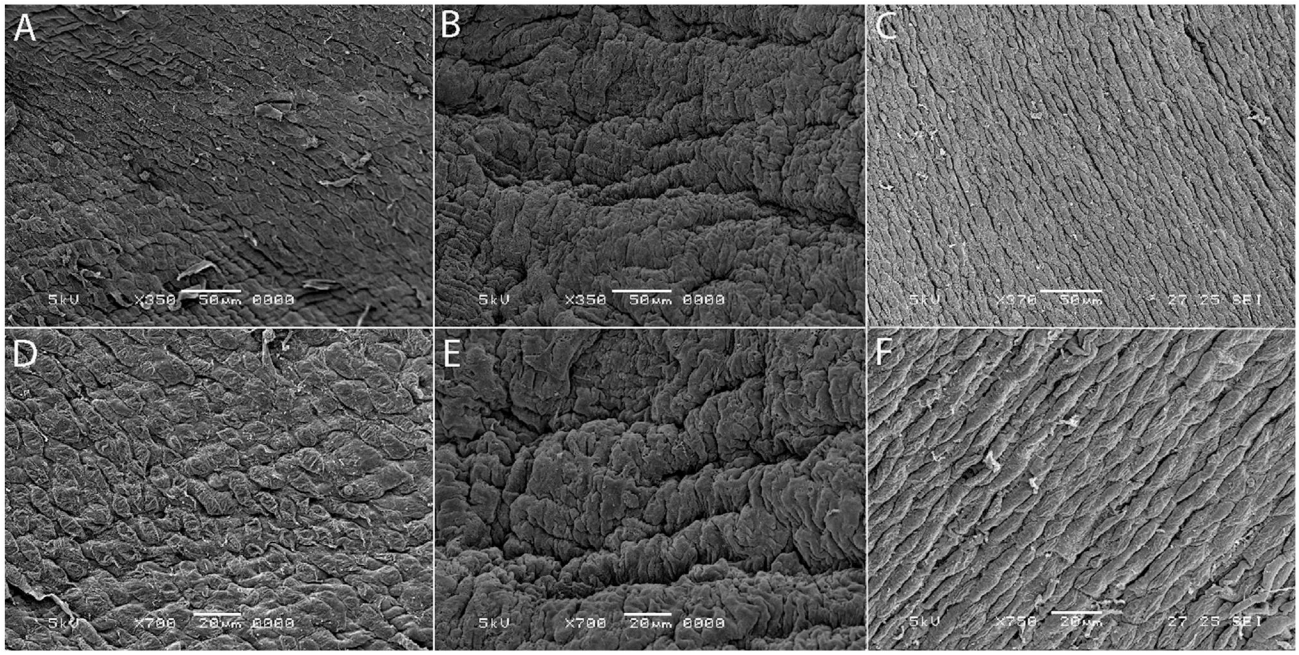

Fig 6.

Scanning electron microscopy analysis of (A, D) fresh samples, (B, E) static preservation at 7 days, and (C, F) flow preservation at 7 days.

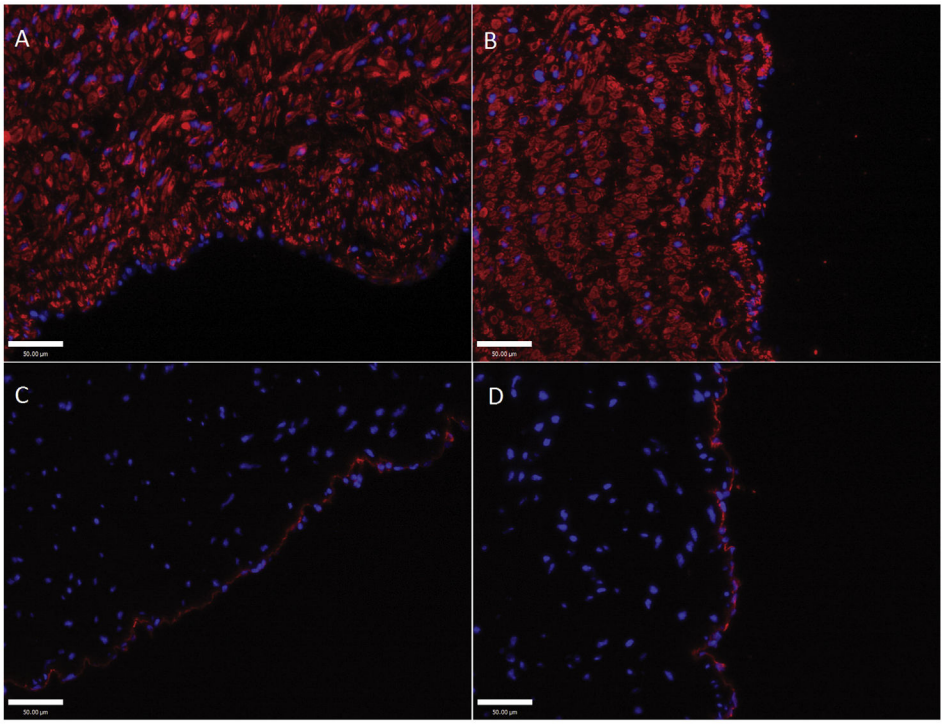

Fig 7.

The α-smooth muscle actin staining for smooth muscle cells with DAPI counterstain of verification samples (A) at delivery and (B) after 7 days of preservation. CD31 staining for endothelium with DAPI counterstain of verification samples(C) at delivery and (D) after 7 days of flow preservation. Scale bar = 50 μm.