Decellularization and Recellularization Methodology for Human Saphenous Veins

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Vascular conduits used during most vascular surgeries are allogeneic or synthetic grafts that often lead to complications caused by immunosuppression and poor patency. Tissue engineering offers a novel solution to generate personalized grafts with a natural extracellular matrix containing the recipient's cells using the method of decellularization and recellularization. We show a detailed method for performing decellularization of the human saphenous vein and recellularization by perfusion of peripheral blood. The vein was decellularized by perfusing 1% Triton X-100, 1% tri-n-butyl-phosphate (TnBP) and 2,000 Kunitz units of deoxyribonuclease (DNase). Triton X-100 and TnBP were perfused at 35 mL/min for 4 h while DNase was perfused at 10 mL/min at 37 °C for 4 h. The vein was washed in ultrapure water and PBS and then sterilized in 0.1% peracetic acid. It was washed again in PBS and preconditioned in endothelial medium. The vein was connected to a bioreactor and perfused with endothelial medium containing 50 IU/mL heparin for 1 h. Recellularization was performed by filling the bioreactor with fresh blood, diluted 1:1 in Steen solution, and adding endocrine gland-derived vascular endothelial growth factors (80 ng/mL), basic fibroblast growth factors (4 µL/mL), and acetyl salicylic acid (5 µg/mL). The bioreactor was then moved into an incubator and perfused for 48 h at 2 mL/min while maintaining glucose between 3 - 9 mmol/L. Later, the vein was washed with PBS, filled with endothelial medium and perfused for 96 h in the incubator. Treatment with Triton X-100, TnBP and DNase decellularized the saphenous vein in 5 cycles. The decellularized vein looked white in contrast to normal and recellularized veins (light red). The hematoxylin & eosin (H&E) staining showed the presence of nuclei only in normal but not in decellularized veins. In the recellularized vein, H&E-staining showed the presence of cells on the luminal surface of the vein.
hold blood pressure. Studies have demonstrated that during damage, endothelialization occurs either from anastomosis or from circulating endothelial progenitor cells (EPCs) in blood. Our strategy for recellularization of veins relies on the EPCs present in circulating blood.

Tissue engineering of veins and arteries was performed by several groups following different decellularization and recellularization strategies. Our group has also performed and developed decellularization and recellularization strategies for iliac and mammary veins. Decellularization was performed by agitation of the vein in Triton X-100, tri-n-butyl phosphate (TnBP), and enzyme deoxyribonuclease (DNase). The recellularization was performed using either bone marrow derived endothelial and smooth muscle cells or peripheral blood. The veins recellularized by either protocol showed clinical promise in providing functional blood supply in transplantation of pediatric patients with extra hepatic portal vein obstruction.

We have currently developed a modified version of the same protocol for the improved and easy performance of decellularization, recellularization and bioreactor handling of small diameter veins. The current decellularization protocol required perfusion of detergents through the vein using pressure instead of agitation with detergents. The recellularization protocol involves an additional step of preconditioning for improving cell adhesion and the addition of growth factors in circulating blood for improving cell adhesion, survival and proliferation. We have also improved the design of the bioreactor using commercially available products. In this paper, we present a detailed description of the modified protocol for performing decellularization and recellularization of human saphenous veins.

Protocol

The tissue used, and the protocol of this paper follows the ethical guidelines of Gothenburg University.

1. Tissue Preparation and Storage

   1. Cut the surrounding tissue from the retrieved saphenous vein using surgical forceps and scissors. NOTE: The saphenous vein is dissected from the lower limb of humans by vascular surgeons. The saphenous vein used in this paper is an un-used part after bypass surgery.
   2. To prevent leakage during perfusion, ligate all the side branches at their ends with sutures and forceps.
   3. Keep the vein in a 50 mL tube containing 40 mL of phosphate buffer saline (PBS). Wash the vein by changing PBS 2 - 3 times to remove excess blood. Store the vein by freezing at -80 °C.

2. Preparation of Decellularization Setups and Recellularization Bioreactor

   NOTE: Using scissors, cut the silicon tubes as shown in Table 1.

   1. Decellularization Setup 1 (For Triton X-100 perfusion and washing)
      1. To a 2 L chamber (plastic tub), tape all three pieces of tube A as shown in Figure 1A.
         NOTE: Tape one tube to one wall where the edge touches the bottom of the chamber (detergent's inlet) and the other two tubes to the opposite wall with a distance of 5 - 10 cm in between depending on the length of the vein. At least 5 cm of these tubes should also be taped to the chamber's floor (vein's inlet and outlet).
      2. Using the male and female Luer connectors, connect in series the detergent's inlet tube to tube B followed by tube F, tube C and finally to the vein inlet. Place the tube F into the cassette of the peristaltic pump I.
      3. Take another tube C and connect one end to the vein's outlet. Place the other end into the glass jar with the bottom hose outlet that is placed 45 cm above the chamber (detergent outlet) and tape it to secure. Take tube G and push one end into the hose outlet of the glass jar and place the other end into the vein chamber.
         NOTE: The pictures of complete setup (red arrows), chamber, and flow direction (white arrows) are shown in Figure 1A.

   2. Decellularization Setup 2 (For TnBP perfusion)
      1. Take a 1 L glass jar, and place one end of tube C (detergent inlet) into the jar until the tube touches the jar's bottom.
      2. Connect the other end to tube F followed by tube C. Place the other end of tube C into the jar until half depth (vein's inlet). Place tube F into the cassette of peristaltic pump I.
      3. Take tube D and place one end into the jar (vein's outlet) until half depth and the other end into the glass jar with the bottom hose placed at a 45 cm height from the vein's inlet (detergent outlet). To secure, tape the detergent's inlet and outlet tubes.
      4. Place the 1 L glass jar on a magnetic stirrer and keep the magnet inside the bottle.
      5. Take tube G and push one end onto the hose outlet of the glass jar and place the other end into 1 L glass jar.
         NOTE: The picture of complete setup (red arrows), chamber and flow direction (white arrows) is shown in Figure 1B.

   3. Decellularization Setup 3 (For DNase perfusion)
      1. Take a 250 mL glass bottle and place both ends of tube C. Place the middle area of the tube into the cassette of peristaltic pump II.
         NOTE: One end of the tube is the solution inlet and the other end of the tube is connected to the vein's inlet. The vein's outlet is left free in solution.
      2. Place the whole setup including pump at 37 °C. The picture of complete setup and flow direction is shown in Figure 1C.

4. Recellularization Bioreactor

   1. Keep ready all the parts as shown in Figure 2A.
      2. As shown in Figure 2B, take the 4-port cap and insert into each port, tube H (vein's outlet), tube I (media inlet), and tube J (vein's inlet). Insert the other end of tube H into the 4th port (media outlet).
         NOTE: The view of the 4-port cap from the top is shown in Figure 2C. For easy insertion of tubes, cut the edges to a sharp point with a scissor.
      3. Bend the other end of tube J into a U shape and tie it with a suture. Connect the reducing connectors to the inner ends of tubes H & J.
4. Place the 60 mL tube with a flat base into the 250 mL glass bottle and then place the above made setup into the tube as shown in Figure 2D. As shown in Figure 2E, connect tubes K, E, and K in series. Connect one tube K to the vein's inlet and another tube K to the media inlet.

NOTE: The schematic diagram of the setup, and flow direction is seen in Figure 2F.

5. Sterilize the bioreactor by autoclaving.

3. Preparation of Solutions

1. Solution 1 (10x water): To 1 L of ultrapure water, add 2 g of sodium azide and 18.6 g of ethylenediaminetetraacetic acid (EDTA). Stir until salts are dissolved.

2. Solution 2 (1x water): Take 100 mL of solution 1 and add 900 mL of ultrapure water.

3. Solution 3 (5x Triton X-100): To 950 mL of ultrapure water, add 50 mL of Triton X-100, 1 g of sodium azide, and 9.3 g of EDTA. Stir until dissolved.

4. Solution 4 (1x Triton X-100): Take 200 mL of solution 3 and add 800 mL of ultrapure water.

5. Solution 5 (1x TnBP): Add 10 mL of TnBP with a 10 mL pipette to 990 mL of solution 2.

6. Solution 6 (40 Kunitz units/mL DNase): Add 2,000 Kunitz units of DNaseI in 50 mL of Dulbecco's Phosphate Buffer Saline containing CaCl$_2$ and MgCl$_2$. Prepare fresh and use immediately.

7. Solution 7 (PBS): Add 24 g of sodium chloride, 0.6 g of potassium chloride, 4.32 g of sodium phosphate, and 0.72 g of potassium phosphate to 3 L of ultrapure water. Stir until dissolved. Adjust the pH to 7.4. Sterilize 1 L of PBS in the autoclave and store separately.

8. Solution 8 (0.1% Peracetic acid): To 50 mL of sterile solution 7, add 50 µL of peracetic acid. Prepare fresh and use immediately.

4. Preparation of Endothelial Medium

1. Add 5 mL of L-glutamine, 5 mL of anti-anti, 50 mL of human AB serum and all components of EGM-2 growth factor kit except fetal bovine serum to 500 mL of the MCDB131 medium under a sterile hood.

5. Decellularization of Saphenous Vein

1. Thaw the human saphenous vein from -80 °C at room temperature. Freeze again at -80 °C and thaw again at room temperature.

2. Cut a biopsy of 2 mm length using scissors and fix in formaldehyde for 24 - 48 h at room temperature. Tie each end of the vein to the barbs of a male and female Luer connector with a suture.

3. Connect the vein to decellularization setup 1 and fill 1 L of solution 2. Perfuse for 15 min at 35 mL/min. Empty the contents of the setup.

4. Add 1 L of solution 4 and perfuse for 4 h at 35 mL/min. Empty the contents of the setup.

5. Add 500 mL of solution 2 and perfuse for 5 min. Empty the contents of the setup. Repeat this step. Disconnect the vein from perfusion setup 1 and connect to perfusion setup 2.

6. Add 1 L of solution 5, turn on the stirrer and perfuse for 4 h at 35 mL/min.

NOTE: Solution 5 looks cloudy after turning on the stirrer and throughout perfusion.

7. Disconnect the vein from perfusion setup 2 and wash the vein's outside and inside with syringe or a 10 mL pipette in 4 changes of ultrapure water for 5 - 10 min.

8. Connect the vein to perfusion setup 3, add 50 mL of solution 6 and perfuse at 10 mL/min in a 37 °C chamber for 4 h. Empty the contents of the setup and disconnect the vein from the setup.

9. Connect the vein to perfusion setup 1, fill 1 L of solution 2 and perfuse overnight at 35 mL/min. Repeat step 5.4 to step 5.9 4 times (for a total of 5 cycles). Take a biopsy again as stated in step 5.2.

NOTE: Skip step 5.8 second part in cycles 1 and 3.

10. Empty the contents of the setup. Fill 1 L of solution 2 and perfuse for 24 h at 35 mL/min. Change solution 2 after 12 h. Empty the contents of the setup. Fill 1 L of the ultrapure water and perfuse for 24 h at 35 mL/min. Change the ultrapure water after 12 h. Empty the contents of the setup. Fill 1 L of solution 7 and perfuse for 24 h at 35 mL/min. Change solution 7 after 12 h.

6. Verification of Decellularization

1. Wash the formalin fixed biopsies in ultrapure water for 15 min. Process in a tissue processor following standard protocols and embed in paraffin$^{22}$.

2. Cut 5 µm sections using a microtome and stain with Meyer's hematoxylin and 0.2% alcoholic eosin (H&E) following standard protocols$^{22}$.

3. View under a microscope to check for loss of nuclei in decellularized tissue compared to normal tissue.

7. Recellularization

1. Sterilize the vein by placing it in a 50 mL tube containing 50 mL of solution 8. Agitate at 80 rotations per minute (rpm) in the shaker for 1 h at 37 °C.

2. Handle the vein under the laminar flow (LAF) cabinet from now on to maintain sterility. Transfer the vein using sterile forceps to a new 50 mL tube containing 50 mL of sterile solution 7 and 500 µL of anti-anti. Agitate as above for 12 h. Change Solution 7 at least twice in between.

3. Using sterile forceps, transfer the vein to a new 50 mL tube and add 50 mL of endothelial media. Agitate as above for 11 h.

4. Assemble the bioreactor using sterile gloves under the LAF cabinet. Tie the vein to the bioreactor's vein inlet and outlet with sterile suture and forceps.

5. Connect the vein to the bioreactor and fill with the same medium. Add heparin at 50 IU/mL and perfuse for 1 h at 2 mL/min in 37 °C.
6. Collect 15 - 25 mL of fresh blood (depending on the vein's length) from the donor in heparin coated vacutainer tubes and dilute 1:1 with Steen solution. Later, add endocrine gland-derived vascular endothelial growth factor (EG-VEGF, 80 ng/mL), basic fibroblast growth factor (b-FGF, 4 µL/mL) and acetyl salicylic acid (5 µg/mL).

7. Move the bioreactor to a 37 °C incubator with 5% CO₂ and perfuse for 48 h at 2 mL/min.

8. Approximately after 12 h, move the bioreactor to a LAF cabinet, take a drop of blood and measure glucose using a blood glucose monitoring device. If the level is less than 3 mmol/L, add glucose to bring in the range of 7 - 9 mmol/L. Repeat this step every 8 - 12 h.

9. After 48 h, move the bioreactor into the LAF cabinet and drain the blood from the bioreactor. Add 30 mL of sterile solution 7 and perfuse for 5 min. Drain the solution. Add 30 mL of sterile solution 7 and perfuse for 5 min. Repeat twice or until the blood red color is lost.

NOTE: The biopsy can be taken for verification of the cell attachment. Disconnect the vein, cut 5 mm edges of the vein with scissors and discard them. Take a biopsy as stated in step 5.2 and connect the vein again to the bioreactor (optional step).

10. Add 30-45 mL of endothelial medium and perfuse for 96 h in the incubator at 2 mL/min.

NOTE: Add endothelial medium until the vein is submerged.

11. Move the bioreactor into the LAF cabinet, disconnect the recellularized vein, cut 5 mm edges of the vein using scissors and discard. Take a biopsy as stated in step 5.2.

8. Verification of Recellularization

1. Follow instructions in section 6 and perform H&E staining. Examine the slides under a microscope for the presence of cells.

Representative Results

The gross morphology of a normal vein is light red (Figure 3A). The red color is lost in progressive decellularization cycles (cycle 2, Figure 3B; cycle 3, Figure 3C) and by the 5th cycle, it looks pale and white (Figure 3D). The recellularized vein after blood perfusion (Figure 3E) and endothelial media perfusion (Figure 3F) looks bright red in color. The 5 cycles of decellularization treatment successfully removed the cells from the vein as no blue nuclei in the H&E staining (Figure 4B) were seen. In contrast, several nuclei were seen in the normal vein (Figure 4A). The presence of attached cells on the luminal side is seen in H&E staining in the recellularized vein with blood for 48 h (Figure 4C, black arrows) and after perfusion with the endothelial medium for 96 h (Figure 3D, black arrows).

Figure 1: Assembling of perfusion setups for decellularization. A) The picture showing the assembled decellularization setup 1 for Triton X-100 and washing. The white arrows show the flow path for solutions and red arrows indicate inlets and outlets for vein and solutions. B) The picture showing the assembled decellularization setup 2 for TnBP perfusion. Similarly, as in decellularization setup 1, white arrows indicate the flow path for solutions and red arrows indicate inlets and outlets for vein and solutions. C) The picture showing the assembled decellularization setup 3 for deoxyribonuclease perfusion. The white arrows show the flow path for solutions and red arrows indicate inlets for vein and solutions. Please click here to view a larger version of this figure.
Figure 2: Preparation and assembling of the bioreactor for recellularization. A) Picture showing materials required for assembling the bioreactor. B) Picture of the inside of 4-port cap showing the placement of reducing connectors (red arrows), points to connect vein inlet and outlet (white arrows) and arrangement of tubes H, I and J. The free end of tube H is placed into the bioreactor to return media. C) The respective tubes going in and out can be seen from the top side of 4 port cap. D) Picture showing the assembled bioreactor. E) Picture showing the whole bioreactor setup with the peristaltic pump. The tubes K extend connections from the bioreactor to the peristaltic pump. F) The schematic representation of whole bioreactor perfusion system. The orange arrows indicate the direction of flow. Please click here to view a larger version of this figure.

Figure 3: Gross morphology of veins during decellularization and recellularization. A) The gross morphology of normal vein looks bright red in color. The color is lost with increasing numbers of decellularization cycles B) cycle 2 and C) cycle 3. D) By 5 cycles, the vein looks pale and white. The vein after perfusing with E) blood for 48 h and F) with the endothelial medium for 96 h looks once again bright red in color. Please click here to view a larger version of this figure.
Figure 4: Characterization of decellularized and recellularized veins. The hematoxylin and eosin staining image of A) normal vein contains many blue nuclei but they are absent in B) decellularized vein. In the vein recellularized with C) blood for 48 h and with D) endothelial medium for 96 h, attachment of cells (black arrows) at lumen was noticed. Please click here to view a larger version of this figure.

Discussion

The technique presented here for decellularization of saphenous veins is an easy, simple and cost-effective method that can also be applied to all small diameter veins like umbilical and mammary veins. The decellularization solutions and their concentrations used in this method are from our previous results\(^6,7\). Even though we recommend 5 cycles of decellularization, in certain veins we also noticed complete decellularization in 3 cycles. However, reproducible results were obtained by using 5 cycles. Applying this protocol, we decellularized veins of varying lengths up to 30 cm successfully (unpublished result). Lifting the decellularization solution’s outlet by 45 cm will create a pressure of 33 mmHg inside the vein. In our experience, we noticed this as a key step in decellularizing the whole vein uniformly and reproducibly in 5 cycles. The chosen pressure is 3 times higher than the normal saphenous vein pressure (5 - 10 mmHg) but is the same as in incompetent veins (varicose veins)\(^23\). In addition, we speculate that this high pressure will create a significant force on vein walls and may, therefore, aid in efficient and faster cell removal.

Since TnBP is an organic solvent and insoluble in water, stirring the detergent until it becomes cloudy is important; otherwise, the detergent will float. For the same reason, to keep TnBP mixed in solution, the detergent's outlet tube was placed at the top of the glass jar with the hose outlet. Efficient removal of TnBP from the vein after its usage in every cycle can be seen by the absence of floating TnBP droplets in the washed water. We have also noticed that skipping the DNase step also produced a decellularized tissue but in a few cases, a comparatively high DNA content in the decellularized tissue was noticed. As high pressure and high flow rates are not required for efficient DNase activity, a low perfusion rate (10 mL/min) may be used. A different peristaltic pump was used as its smaller size helps in easy handling of the setup. Since we noticed that damage of most cells during decellularization results in cycle 2, we suggest skipping DNase treatment during cycles 1 and 3 (unpublished result). Even though the characterization and quantification of extracellular matrix proteins were not performed in this manuscript, our previous experience with similar decellularization protocols showed the preservation of biomechanical properties, extracellular matrix structure and proteins\(^7\). Though our preliminary quantification experiments with these veins produced a similar result (unpublished), our already published results will strengthen this confidence.

Recellularization using blood is a convenient and easy process over bone marrow expanded cells as one can avoid long cell expansion times, spontaneous mutations in expanded cells, surgical invasion, and discomfort for the patient. Since it is known that endothelialization can also occur from circulating EPCs, we hypothesized that perfusion with blood followed by perfusion with endothelial medium will be sufficient for recellularization. The safety of vein tissues engineered using a similar method is seen from successful results of transplantation when two such veins were implanted into children with extra hepatic portal vein obstruction\(^7\). We speculate that the growth factors in decellularized extracellular matrix will permit the attachment of circulating EPCs from blood\(^24\). Recellularization of veins following a similar protocol showed cells positive for VEGF receptor-2 and cluster of differentiation (CD) 14 on the lumen while CD45 expressing cells were seen in adventitia\(^7\). We also imagine that a continuous endothelial layer may not be observed in all cases especially when using blood from older and diseased patients as it is known that such individuals have decreased numbers of circulating progenitor cells\(^25\). However, we postulate that perfusion with the recipient's own blood may mask many of the antigens that are exposed because of decellularization and may thus decrease the chances of adverse inflammatory reactions when transplanted as opposed to transplanting only decellularized blood vessels. In addition, blood perfusion can deposit increased levels of growth factors on the lumen and adventitia which in turn may recruit increased numbers of circulating progenitor cells resulting in a rapid process of recellularization in vivo.
The advantages of the bioreactor design used in this study are complete autoclaving, easy assembly, cost-effectiveness, easy handling and the least possibility of damage. In our experience, using the current design, veins up to 10 cm in length were recellularized. Even though veins up to 25 cm in length can also be recellularized by keeping the vein in "U" shape inside the bioreactor, this should be validated. The bioreactor design shows that the direction of flow in the vein is against gravity and is designed as such because it is the normal direction of flow for these veins in humans. The 12 h perfusion of the endothelial medium step is to precondition the vein and increase the affinity for attachment of incoming EPCs. Addition of extra heparin and perfusion for 1 h will lower the risk of forming blood clots in the tubes during blood perfusion.

The volume of blood required is dependent on the length of the vein. The basic principle we follow for blood volume is that the vein should be submerged in blood. While handling blood volumes larger than 45 mL, occasional mixing might be required to prevent cell accumulation in the bottom of the bioreactor. We added Steen's solution to blood since it contains a high amount of proteins and components required to maintain tissues healthy during organ transplantation. Addition of VEGF and b-FGF is beneficial as they are potent angiogenic growth factors and their presence induces migration, proliferation, and differentiation of EPCs. The amount of VEGF added is based on our previous unpublished results where the proliferation of EPCs was seen at 80 ng/mL. Addition of aspirin inhibits the activation of platelets thereby decreasing the chances of their attachment to endothelial layer. Continuous monitoring and addition of glucose will also be beneficial for cell proliferation and preventing hemolysis of red blood cells.

Since only a simple blood sample is required from the recipient, it can be considered as an easy and feasible procedure requiring less technical expertise. Even though, the whole procedure shown here from start to finish takes 20 days, storing the decellularized veins as an off the shelf product will shorten the procedure to 8 days for patients. Although storage of decellularized veins technically should not affect the recellularization efficiency, it must be evaluated. Tissue-engineered veins generated following this procedure can be used in the clinic for bypass surgeries, replacing obstructed veins, venous insufficiency leading to varicose veins without the need for immune suppression and thus providing a better quality of life for the patient.

Disclosures

SSH holds shares in Verigraft, a company that has licensed the technology of tissue engineering blood vessels. The other authors have nothing to disclose.

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