In vivo tissue engineering of a trilayered leaflet-shaped tissue construct

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Aim: We aimed to develop a leaflet-shaped trilayered tissue construct mimicking the morphology of native heart valve leaflets. **Materials & methods:** Electrospinning and *in vivo* tissue engineering methods were employed. **Results:** We developed leaflet-shaped microfibrinous scaffolds, each with circumferentially, randomly and radially oriented three layers mimicking the trilayered, oriented structure of native leaflets. After 3 months *in vivo* tissue engineering with the scaffolds, the generated leaflet-shaped tissue constructs had a trilayered structure mimicking the orientations of native heart valve leaflets. Presence of collagen, glycosaminoglycans and elastin seen in native leaflets was observed in the engineered tissue constructs. **Conclusion:** Trilayered, oriented fibrous scaffolds brought the orientations of the infiltrated cells and their produced extracellular matrix proteins into the constructs.

Graphical abstract:

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Heart valve diseases are major health problems worldwide [1,2]. Prosthetic valves – mechanical valves or bioprosthetic valves – are the only options for survival of patients. However, these valves face several limitations: mechanical valves require lifelong application of anticoagulants and bioprosthetic valves require sequential surgeries as they degrade and calcify after a limited period [3,4]. Further, none of them can be used for pediatric patients as they cannot grow or remodel with the patients.

Developing a tissue-engineered heart valve could be a potential solution to the problems associated with prosthetic valves [5–7]. Tissue functionalities depend on the tissue structures. Thus, a tissue-engineered heart valve should mimic the structure of a native heart valve to achieve native functionalities. In a native heart valve, the structure of its leaflets is most complicated and consists of three layers: a circumferentially oriented fibrosa layer, a randomly oriented spongiosa layer and a radially oriented ventricularis layer [4,8–10]. To develop a functional tissue-engineered heart valve, it is important to produce leaflet-shaped trilayered tissue constructs mimicking the structure of a native leaflet. In this study we made an effort to develop native leaflet-mimicked tissue constructs.

Tissue engineering can be performed *in vitro*, *in vivo*, *in situ* or in combination. *In vitro* tissue engineering is generally accomplished by seeding cells in a biomaterial scaffold and then culturing/incubating that seeded scaffold in a static and/or dynamic environment. However, *in vivo* tissue engineering is much more efficient than *in vitro* tissue engineering to generate a robust neotissue, especially a neoleaflet tissue that can withstand physiological pressure [11,12]. Further, an autologous tissue construct can be developed with minimal effort through *in vivo* tissue engineering. Our ultimate goal is to develop an autologous heart valve tissue through *in vivo* (subcutaneous) tissue engineering and use that tissue-engineered valve for heart valve replacement. Thus, development of autologous trilayered leaflet tissues through *in vivo* tissue engineering was part of the goal. Several researchers made efforts to develop autologous heart valves through *in vivo* tissue engineering by implanting the heart valve molds (without any scaffold) subcutaneously [11,12]. But lack of any native structure in the tissue-engineered heart valves may cause functional limitations over time because residing valvular interstitial cells are very sensitive to the structure of the constructs and may bring adverse effect due to absence of the native structure [13,14]. We have been trying to advance the autologous tissue constructs by incorporating the native structure in them.

Previously, various researchers developed trilayered scaffolds (TS) aiming to generate trilayered leaflets through tissue engineering. The scaffolds were made of fibrous, hydrogel, microstructure materials or combinations of them [4,9,10,13]. For example, Masoumi *et al.* developed trilayered structures with a fiber–microstructure–fiber combination of polycaprolactone (PCL) and polyglycerol sebacate polymers [4]. In another approach, TSs were prepared from a synthetic hydrogel – polyethylene glycol diacrylate hydrogel – with appropriate mechanical properties in each layer by controlling the cross-linking density of the polyethylene glycol diacrylate hydrogel [10]. Aligned collagen fibrils were also used to fabricate the outer layer of TSs [9]. Our TSs will be made of microfibers mimicking the orientations of the layers in native leaflets. We opted for fibrous scaffolds as fibers in the scaffolds mimic the fibril morphology of extracellular matrix (ECM) in native leaflets. Thus, cells inside the scaffolds would receive appropriate morphological environment during tissue engineering and a scaffold with a fibrous morphology will have higher degree of orientation of the deposited proteins compared with the scaffolds with other morphologies [15,16]. Our lab is the first to develop leaflet-shaped, trilayered fibrous scaffolds mimicking the structure and orientations of a native heart valve leaflet.

PCL was used to fabricate leaflet-shaped trilayered fibrous scaffolds through electrospinning. PCL, a biocompatible and biodegradable polymer, is easy to electrospin [17,18]. Devices made of PCL do not face difficulties in securing US FDA approval; thus an electrospun fibrous scaffold made from PCL can be used for *in vitro/in vivo* tissue engineering. Further, mechanical properties and biodegradability of PCL scaffolds can be varied by changing the molecular weight of PCL [19].

Microfibers can be electrospun from PCL solution at high concentration (22 wt/v%) [20]. Microfibers have an advantage over nanofibers for *in vivo* tissue engineering. During *in vivo* tissue engineering, M1 phenotype macrophage cells migrate to encourage inflammation and M2 phenotype macrophage cells migrate to encourage tissue repair [21,22]. Compared with nanofibers, microfibers attract more M2 macrophage cells and thus, facilitate tissue growth [23]. Further, pore sizes are higher in a scaffold made of microfibers than in a scaffold made of nanofibers, i.e. microfibrillar scaffolds are more efficient than nanofibrillar scaffolds for cell infiltration or penetration during *in vivo* or *in vitro* tissue engineering [24].

In this study, we developed leaflet-shaped trilayered fibrous scaffolds mimicking the orientations in native valve leaflets. Then, we performed *in vivo* tissue engineering of leaflet-shaped tissue constructs by implanting the leaflet-shaped fibrous scaffolds in a rat model subcutaneously. After 3 months of implantation, explanted tissue constructs were characterized structurally, mechanically and biologically to find the effect of the trilayered fibrous structure on *in vivo* tissue engineering in terms of orientations of cells and their deposited ECM, tensile properties of the constructs and presence of different ECM proteins including collagen, glycosaminoglycans (GAG) and elastin.

Materials & methods

Leaflet-shaped collector fabrication

A leaflet-shaped collector was designed using CAD/CAM software. By applying the design in a 3D printer, several leaflet-shaped collectors were produced from a nonbiodegradable biomaterial. Each collector consisted of three surfaces – leaflet-shaped surface, aortic sinus-shaped surface and base surface, and two cylindrical holes. These holes were used as axes of rotations of the collector.

Leaflet-shaped scaffold fabrication

A 22% (wt/v) PCL (MW: 80 KD, Sigma-Aldrich, WI, USA) solution in chloroform was used to electrospin (parameters – voltage: 15 kV, solution flow rate: 0.03 ml/min and spinneret-collector distance: 10 cm) fibers for scaffold development. A leaflet-shaped collector was metal-coated using a sputter-coater to make its surface electrically conductive. The collector was rotated for 20 min at 2000 r.p.m. with respect to the axis on the base surface in front of the spinneret of an electrospinning system to deposit circumferentially oriented fibers on the collector. Then, the collector was rotated for 15 min at 50 r.p.m. with respect to the same axis to deposit randomly oriented fibers. After that, the collector was rotated for 10 min at 2000 r.p.m. with respect to the second axis to deposit radially oriented fibers. After depositing three layers of fibers, a leaflet-shaped, TS was removed from the collector.

Leaflet-shaped mold fabrication & implant preparation

A leaflet-shaped mold was designed using CAD/CAM software. The mold consisted of two parts – a leaflet-shaped core-structure and a leaflet-shaped cover. The cover could fit to the core-structure with a set of four holes and four pins. By applying a 3D printer, several core-structures and their covers were produced from a nonbiodegradable biomaterial. When a core-structure and its cover were set, they together made a leaflet-shaped cavity where a leaflet-shaped fibrous scaffold could be inserted. To prepare an implant, a leaflet-shaped, TS was placed on the leaflet-shaped surface of a core-structure and then a cover was fitted to the core-structure with hole-pin sets.

Scanning electron microscopy imaging

Leaflet-shaped scaffolds were processed for scanning electron microscopy (SEM) imaging to find various physical properties including morphology, orientation of fibers, diameter of fibers and pore size. Scaffolds were coated with gold–palladium metal in a sputter-coater and then imaged with a SEM (Hitachi, Japan). Fiber diameters in the SEM images of several samples ($n = 5$) at different spots ($n = 10$) were measured using a slide calipers (Mitutoyo, Kawasaki, Japan) and their average was calculated. Thickness of scaffolds ($n = 5$) at different spots ($n = 7$) was measured using a gauge (Mitutoyo) and their average was calculated. Scaffolds were imaged cross-sectionally to measure the thickness of each layer.

Implantation & explantation

A total of two samples – one test sample and one control sample, were implanted in the dorsal area of each rat (Sprague-Dawley, 1–2 months, ~250 gm), subcutaneously. A test implant sample had the scaffold inside the cavity of a mold whereas a control implant sample was only a mold (without any scaffold). Implant samples (both test and control) were soaked with blood collected from the tail vein of rats. A 3-cm incision was made at the dorsal area below the chest of a rat with a sterile scalpel blade. Skin at two sides of the incision was loosened with a blunt dissection to make two subcutaneous pockets in which one test sample and one control sample were placed. The incision was then sutured. A total of six rats (i.e., six test samples and six control samples) were used for *in vivo* tissue engineering. After 3 months post-implantation, the samples were carefully explanted. The engineered tissue samples (test and control) were removed from the molds and characterized. This study was performed in accordance with authorization and guidelines of the Ethical Committee of Mayo Clinic, MN, USA.

Transmission electron microscopy imaging

Tissue samples were fixed in 10% formalin for 24 h. The samples were then processed following the protocols described in our previous published papers and then embedded in epoxy resin [13,16]. The embedded samples were cut into thin sections that were collected on copper grids and imaged with a transmission electron microscope (Jeol, Tokyo, Japan).

Tensile testing

To perform tensile tests on TS, test tissue constructs or control tissue constructs, two paper window-frames with window dimensions of 5 mm × 4 mm were made, a rectangular-shaped scaffold or tissue sample with dimensions of 9 mm × 3 mm was cut from a big sample keeping either circumferential or radial orientation as axis of tensile loading, sandwiched between two window-frames and glued (super glue) to prepare a sample for tensile test. Tensile tests were performed along either circumferential orientation or radial orientation. The samples were loaded at a rate of 0.1 mm/s. A total of five samples (n = 5) of each type in each direction were used for tensile tests.

Histology & immunohistochemistry staining

Tissue samples were fixed in 4% paraformaldehyde and then embedded in paraffin to prepare tissue sections for staining. The sections were stained histologically with hematoxylin and eosin (H&E; Thermo Fisher Scientific, MO, USA), Masson's trichrome (MT; ScyTek Lab, UT, USA), picrosirius red (ScyTek Lab) and Safranin O (ScienCell Research Lab, CA, USA) separately. Tissue sections were also assayed immunohistochemically with elastin (ab21610, Abcam, CA, USA), vimentin (ab92547, Abcam), α -SMA (ab124964, Abcam), INOS (ab3523, Abcam) and CD206 (orb4941, Biorbyt, MO, USA) antibody markers separately. For staining of tissue sections, protocols published in our previous papers were used [13,16].

Using ImageJ (NIH, MD, USA) software and following a procedure detailed in our previous studies, fast Fourier transform (FFT) was performed on the histology images of the collagen fibrils in the trilayered tissue constructs to determine the degree of alignment of the fibrils [25,26]. In brief, a histology image was first transformed into FFT intensity image. The intensity image was then normalized to a vertical axis with a baseline value of zero. By applying an oval-profile plug-in, the radial intensity was summed and plotted against the angle of acquisition.

Quantification using image

Signals in histological and immunostained images were quantified using MetaMorph software. Images of 20× magnification were used for quantification. A threshold color-intensity from all staining images of a particular protein or cell to be quantified was chosen. Percentage of number of pixels with this threshold intensity (or more) with respect to the total number of pixels of an image was measured. The test sample data were normalized against control sample data.

Statistical analysis

Mean $\pm$ standard deviation was used to report the data. An unpaired t-test for two-group comparisons was conducted. A one-way ANOVA with Tukey's *post-hoc* test for three-group comparisons was conducted. $p < 0.05$ were applied to indicate significance.

Results

Trilayered scaffold fabrication

A leaflet-shaped collector was designed and produced in a 3D printer (Figure 1A–C). The collector had three surfaces – a leaflet-shaped surface (Figure 1A), a sinus-shaped surface (Figure 1B) and a base surface (Figure 1C). The collector also had two hole-axes – one on the base surface and another on the sinus-shaped surface, representing two axes of rotations (shown by black arrows). The collector was made of a plastic material and was coated with a metal alloy in a sputter coater to make its surfaces electrically conductive (Figure 1D–F). To develop a fibrous scaffold, a 22% PCL solution in chloroform was electrospun on the rotating collector. First, fibers were deposited on the collector rotated at 2000 r.p.m. around its hole-axis on base surface for 20 min to develop a circumferentially aligned fibrous layer (Figure 1G). Then, the collector was rotated for 15 min at 50 r.p.m. around the same axis to deposit randomly oriented fibers on the circumferential layer (Figure 1H). The collector was then rotated for 10 min at 2000 r.p.m. around its hole-axis on sinus-shaped surface to deposit a radially aligned fibrous layer on the deposited bilayer (circumferential and random layers) (Figure 1I). Thus, three layers of fibers were deposited on the collector (Figure 1J). Three different periods were applied to achieve three layers with three thicknesses, respectively. Using a sharp blade, a leaflet-shaped scaffold was taken off from the deposited whole scaffold on the collector and characterized. Inner circumferential layer and outer radial layer of the scaffold are shown in Figure 1K & L, respectively. Orientations of fibers in these layers are shown by the double-headed arrows.

SEM images of the scaffolds were used to verify the alignments of fibers in three layers of the scaffolds. Images were taken both at low and high magnifications to observe the alignments of fibers accurately. At low magnification,

Figure 1. Trilayered, oriented scaffold fabrication. (A–C) A leaflet-shaped collector showing different surfaces and two hole-axes: (A) a leaflet-shaped surface, a sinus-shaped surface with a hole-axis (shown by an arrow (B) and a base surface with a hole-axis (shown by an arrow; C). (D–F) The leaflet-shaped collector coated with a metal showing different surfaces and two hole-axes. (G–I) Rotation of the collector with respect to two hole-axes: rotation around its axis on base surface (G), rotation around its axis on base surface (H) and rotation around its axis on sinus-shaped surface (I). (J) Three layers of oriented fibers deposited on the collector to form a leaflet-shaped scaffold. (K) The inner (concave) circumferentially oriented (shown by a double-headed arrow) layer of the scaffold. (L) The outer (convex) radially oriented (shown by a double-headed arrow) layer of the scaffold.

Figure 2. Scanning electron microscopy images of fibers in three oriented layers – circumferential, random and radial – in the trilayered, oriented leaflet-shaped scaffolds. (A) Lower magnification and (B) higher magnification.

fibers in the circumferential and radial layers were aligned, and those in the random layer were randomly oriented (Figure 2A). Apparently, fibers in the circumferential layer were more aligned than in the radial layer. At higher magnification, alignment and randomness of fibers in respective layers were more distinct (Figure 2B). Fiber diameter was in the range of 5–8 μm . It can easily be observed from the images that pore size in all three layers of the scaffolds was more than 10 μm – the general cell size. Thus, the scaffolds had sufficient pore size for cell infiltration during *in vivo* tissue engineering. SEM images of scaffold cross-sections were used to measure the thickness of each layer in scaffolds; thicknesses of circumferential, random and radial layers in scaffolds were ~ 250 , ~ 200 and ~ 175 μm , respectively.

Mold development, implant sample preparation & implantation

For *in vivo* tissue engineering, a mold was designed and produced in a 3D printer. The mold consisted of two parts – a leaflet-shaped core-structure and a leaflet-shaped cover (Figure 3A & B). The core-structure and the cover had four holes and four pins at their peripheries, respectively. With these pins and holes, the cover could

Figure 3. Mold fabrication, implant preparation and explanted tissue construct (n = 6, each type). (A & B) A mold having two parts – a leaflet-shaped cover with four pins (shown by black arrows) and a leaflet-shaped core structure with four holes (shown by white arrows), shown from different views. (C & D) A leaflet-shaped cover fitted to a leaflet-shaped core-structure to form a mold with a mold-cavity. Mold shown from different views. (E & F) A leaflet-shaped trilayered, oriented scaffold sample inserted into the mold-cavity to prepare an implant sample shown from different views. (G) A blood-soaked implant sample. (H) An explant sample after *in vivo* tissue engineering. (I) An *in vivo* tissue-engineered construct developed with a trilayered, oriented leaflet-shaped scaffold. (J) An *in vivo* tissue-engineered construct developed without any scaffold.

be attached to core-structure tightly to make a leaflet-shaped cavity in which a leaflet-shaped scaffold could be inserted (Figure 3C & D). A leaflet-shaped scaffold sample was inserted into the mold cavity to prepare a test implant sample (Figure 3E & F). A mold without a scaffold was designated as a control implant sample. Implant samples including the scaffolds inside the mold cavities were soaked with blood collected from the tail veins of rats (Figure 3G). A total of six test and six control implant samples were implanted in six rats, in other words, each rat had one test and one control implant samples.

Explantation & morphology of the tissue constructs

After 3 months of implantation, the samples were explanted (Figure 3H). Cells infiltrated into the mold cavities and produced ECM proteins to build tissue constructs (Figure 3I & J). Tissue construct samples were gently removed from the mold cavities and processed for their characterization. H&E-stained scaffold-based test tissue constructs showed a trilayered structure – a circumferentially oriented layer (x), a randomly oriented layer (y) and a radially oriented layer (z), forming trilayered tissue constructs, whereas control tissue constructs did not have any layer structure or any alignment (Figure 4A). Orientation in the radial layer is perpendicular to paper; so, only dots are seen in the images. MT staining also showed the trilayered structure in the scaffold-based test tissue constructs and random orientation in the control tissue constructs (Figure 4A). Unlike test tissue constructs which had a leaflet shape, the control tissue constructs were a lump of tissues and were not stretched properly during paraffinization, so gaps occurred among the tissue layers. These gaps were observed in their staining images. In H&E- and MT-stained samples, among other protein materials, mainly collagen fibrils were stained. Infiltrated cells were oriented along the fibers in the scaffolds and their deposited collagen fibrils were oriented accordingly. To find their degree of alignment, ImageJ software was applied on the aligned (circumferentially oriented) layer of H&E-stained images. The peak on the plot is narrow and sharply outlined indicating a high degree of alignment of collagen fibrils (Figure 4B).

To further confirm the trilayered structure in the test tissue constructs, tissue sections were examined with a transmission electron microscopy. At low magnification, a trilayered structure – the circumferential, random and radial layers (separated by white lines) – was observed (Figure 5A). At higher magnification, collagen fibrils were aligned in the circumferential layer (Figure 5B), randomly oriented in the random layer (Figure 5C) and aligned (dots – perpendicular to the paper) in the radial layer (Figure 5D). Conversely, collagen fibrils were randomly

Figure 4. Morphological characterization of explanted tissue constructs. (A) Images of H&E- and MT-stained trilayered tissue constructs and control tissue constructs. In the trilayered constructs, three distinct layers – a circumferentially oriented layer (x), a randomly oriented layer (y) and a radially oriented layer (z), were observed. In the control constructs, no layers were observed. (B) FFT alignment plot of collagen fibrils in the aligned layers of the trilayered tissue constructs against the angle of acquisition (degree). H&E: Hematoxylin and eosin; MT: Masson's trichrome; FFT: Fast fourier transform.

Figure 5. Transmission electron microscopy images of cross-sections of trilayered and control tissue constructs. (A) Three distinct layers in trilayered tissue constructs (low mag.). (B) Aligned collagen fibrils in the circumferential layer of trilayered tissue constructs (high mag.). (C) Randomly oriented collagen fibrils in the random layer of trilayered tissue constructs (high mag.). (D) Aligned collagen fibrils in the radial layer of trilayered tissue constructs (high mag.). Dots represent the alignment perpendicular to the paper. (E) No layers in control tissue constructs (low mag.). (F) Randomly oriented collagen fibrils in control tissue constructs (high mag.).

oriented throughout the control tissue constructs as shown in Figure 5E (lower magnification) and Figure 5F (higher magnification).

Tensile test

Tensile tests were performed on TS, trilayered test tissue constructs and control tissue constructs to compare their tensile properties (tensile modulus and yield stress; Figure 6). Tensile moduli of TS and trilayered tissue constructs in circumferential direction and control tissue constructs were (6.979 ± 1.782 MPa), (4.173 ± 1.159 MPa) and (1.735 ± 1.010 MPa), respectively. These tensile moduli were significantly different. Their yield stresses were

Figure 6. Tensile properties of trilayered scaffolds, trilayered constructs and control constructs (n = 5, each type). Tensile modulus of TC-circum was lower than that of TS-circum and higher than that of control constructs. Tensile strength (yield stress) of TC-circum was quite similar to that of TS-circum but higher than that of control constructs. Tensile modulus of TC-radial was lower than that of TS-radial. Tensile strength (yield stress) of TC-radial was lower than that of TS-radial but quite similar to that of control constructs. TC-circum: Trilayered constructs in circumferential direction; TC-radial: Trilayered constructs in radial direction; TS-circum: Trilayered scaffolds in circumferential direction, TS-radial: Trilayered scaffolds in radial direction.

(1.225 ± 0.012 MPa), (1.048 ± 0.362 MPa) and (0.420 ± 0.139 MPa), respectively. Yield stresses of both TS and trilayered tissue constructs in circumferential direction were significantly higher than that of control tissue constructs. Tensile moduli of TS and trilayered tissue constructs in radial direction and control tissue constructs were (4.082 ± 0.499 MPa), (2.236 ± 0.382 MPa) and (1.735 ± 1.010 MPa), respectively. Tensile modulus of TS in radial direction was significantly higher than that of trilayered tissue constructs in radial direction. Their yield stresses in radial direction were (0.771 ± 0.261 MPa), (0.422 ± 0.134 MPa) and (0.420 ± 0.139 MPa), respectively. Yield stresses of trilayered tissue constructs in radial direction were quite similar to that of control tissue constructs.

Anisotropy ratios of tensile moduli of TS and trilayered tissue constructs were 1.71 ($6.979/4.082$) and 1.87 ($4.173/2.236$), respectively. Anisotropy ratios of tensile strengths (yield stress) of TS and trilayered tissue constructs were 1.58 ($1.225/0.771$) and 2.48 ($1.048/0.422$), respectively.

Extracellular matrix proteins in tissue constructs

Tissue sections were stained to find the presence of different predominant ECM proteins – collagen, GAG and elastin – that are generally observed in a native leaflet (Figure 7A). For collagen detection, the tissues were stained with picrosirius red and imaged in bright light and polarized light. Three orientations were observed in the trilayered tissue constructs. In polarized light, red color collagen fibrils were observed. In the control tissue constructs, collagen fibrils were randomly oriented and in polarized light, these randomly oriented collagen fibrils had a green color. For GAG detection, the tissues were stained with safranin O and imaged in bright light. Three orientations were observed in trilayered tissue constructs and more GAG presence was in the random and radial layers than in the circumferential layers. Control tissue constructs had randomly oriented GAG throughout. For elastin detection, tissue constructs were assayed with elastin antibody marker. Presence of elastin was observed in both trilayered and control tissue constructs (Figure 7A, lower magnification). Elastin had alignments in circumferential and radial layers of the trilayered tissue constructs and it was randomly oriented in their random layers (Figure 7A, higher magnification). In the control tissue samples, elastin was randomly oriented throughout the tissues. For further comparison, a software-based quantification technique was applied on the images of stained/assayed tissue sections and ECM protein materials were quantified (Figure 7B) Collagen in trilayered tissue constructs was significantly higher than in control tissue constructs (quantified on the images taken with polarized light). Other two proteins in trilayered and control tissue constructs had no significant differences.

Vimentin and α -SMA expression by infiltrated cells in tissue constructs

During subcutaneous *in vivo* tissue engineering, infiltration of different cells including fibroblasts occurs. Inclination toward tissue growth in this tissue engineering can be determined by analyzing the vimentin and α -SMA expression by the infiltrated cells [27,28]. Thus, tissue sections were assayed with vimentin and α -SMA antibody markers immunohistochemically. Vimentin expression by infiltrated cells in both types of tissue constructs was detected (Figure 8A). Vimentin expression was more in the random and radial layers than in the circumferential layers of the trilayered tissue constructs. Vimentin expression in the control tissue constructs was almost regular throughout. When compared, more vimentin expression was observed in control tissue constructs than in trilayered tissue

Figure 7. Characterization of predominant extracellular matrix proteins – collagen, elastin and GAG in tissue constructs. (A) Images of picrosirius red- and Safranin O-stained trilayered tissue constructs and control tissue. Presence of collagen fibrils and glycosaminoglycans was observed in both constructs. In the trilayered construct, three distinct oriented layers were observed. In the control construct, no layers and particular orientations were observed. Anti-elastin antibody-stained immunohistochemical images of a trilayered construct and a control construct (at low and high mags.). Presence of elastin observed in both constructs. In the trilayered construct, three distinct oriented layers were observed. In the control construct, no layers and particular orientations were observed. **(B)** Quantification graph of those extracellular matrix materials in trilayered and control tissue constructs (n = 5, each type). TC: Trilayered construct.

constructs. Similar to vimentin, α -SMA expression by infiltrated cells in both types of tissue constructs was detected (Figure 8B). α -SMA expression in trilayered and control tissue constructs was quite uniform throughout the constructs. For further comparison, a software-based quantification method was applied on the assayed images and vimentin and α -SMA signals were quantified (Figure 8C). Both signals in control tissue constructs were significantly higher than in trilayered tissue constructs.

Presence of macrophage cells in tissue constructs

Among various type of cells, M1 phenotype and M2 phenotype macrophage cells were supposed to infiltrate after implantation. Thus, tissue sections were assayed with INOS (an M1 Phenotype macrophage cell marker) and CD206 (an M2 Phenotype macrophage cell marker) markers. Presence of M1 phenotype cells was observed in both trilayered and control tissue constructs (Figure 9A). More M1 macrophage cells were in random and radial layers than in circumferential layers of the trilayered constructs (TCs). Further, it is visually apparent that more M1 macrophage cells were present in control tissue constructs than in TCs. To confirm that, software-based quantification method was applied on the assayed images and INOS signal was quantified. INOS signal in control tissue constructs was significantly higher than in trilayered tissue constructs (Figure 9B).

Similar to M1 macrophage cells, M2 macrophage cells were observed in both trilayered and controls tissue constructs (Figure 9C). More M2 macrophage cells were observed in random and radial layers than in circumferential layers of the TCs. Further, it is visually apparent that more M2 macrophage cells were present in control tissue

Figure 8. Immunohistochemistry analysis of vimentin and α -SMA expressed by infiltrated cells in tissue constructs. (A) Images of anti-vimentin antibody-assayed trilayered tissue constructs and control tissue constructs (at low and high mags.). In the TC, three distinct oriented layers were observed. Alignment was observed in both circumferential layer and radial layer. In the control constructs, no layers and particular orientations were observed. (B) Images of anti- α -SMA TC and a control construct (at low and high mags.). In the TC, three distinct-oriented layers were observed. Alignment was observed in both circumferential layer and radial layer. In the control construct, no layers and particular orientations were observed. (C) Quantification graph of vimentin and α -SMA expression by the cells in both trilayered and control tissue constructs ($n = 5$, each type). α -SMA: α -smooth muscle actin; TC: Trilayered construct.

Figure 9. Immunohistochemical analysis of INOS and CD206 expressed by macrophage cells in tissue constructs. (A) Images of anti-INOS (a M1 phenotype macrophage cell marker) antibody-assayed images of a trilayered construct and a control tissue constructs (at low and high mags.). In the trilayered construct, three distinct-oriented layers were observed. In the control construct, no layers and particular orientations were observed. **(B)** Quantification graph of M1 (marker: INOS) and M2 (marker: CD206) phenotype signals of macrophage cells present in both trilayered and control tissue constructs ($n = 5$, each type). **(C)** Images of anti-CD206 (a M2 phenotype macrophage cell marker) antibody-assayed tissue of a TC and a control construct (at low and high mags.). In the trilayered construct, three distinct oriented layers were observed. In the control construct, no layers and particular orientations were observed.

constructs than in TCs. To confirm that, software-based quantification method was applied on the assayed images and CD206 signal was quantified. CD206 signal in control tissue constructs was significantly higher than in trilayered tissue constructs (Figure 9B).

Discussion

A native heart valve leaflet comprised of mainly fibrous ECM has three layers – a circumferentially oriented fibrosa layer, a randomly oriented spongiosa layer and a radially oriented ventricularis layer [29,30]. This trilayered structure is formed naturally during development to fulfill the functional requirements of native valve leaflets. Thus, it is essential to include the native trilayered structure in tissue-engineered leaflets to achieve their functional efficiencies. During tissue engineering with a scaffold, cells are aligned along the orientations in the scaffold structure, their deposited ECM follows the orientations and together, they form a tissue construct [13,31]. Thus, to develop a trilayered, oriented tissue structure, a scaffold with similar structure could be useful. In this investigation, we developed a fibrous scaffold to obtain the required oriented, trilayered structure.

The surface of a native leaflet is curvilinear [30]. To mimic that surface in the scaffolds, a collector was made with a leaflet-shaped curvilinear surface. Deposited fibers were aligned by rotating the collector with an appropriate

speed in front of the spinneret in an electrospinning system [32]. Rotating the collector with respect to different axes, at different speeds and for different periods, three layers with different orientations and different thicknesses were generated. Thicknesses of circumferential, random and radial layers in a native leaflet are generally 45%, 35% and 20% of the thickness of the leaflet, respectively [4]. Thicknesses of circumferential, random and radial layers in developed scaffolds were ~ 250 , ~ 200 and ~ 175 μm , respectively; i.e., ~ 40 , ~ 32 and $\sim 28\%$ of the thickness of the scaffolds, respectively. By changing the periods used for electrospinning different layers, thickness of each layer in the scaffolds can be controlled and similar to that of respective layers of a native leaflet. Fibers in the scaffolds were circumferentially, randomly and radially oriented mimicking the orientations in a native heart valve leaflet. Fiber diameter in the leaflet-shaped, trilayered fibrous scaffolds was in microscale and pore size was more than 10 μm – these characteristics favored cell infiltration and tissue growth [24].

The scaffolds were implanted in a rat model for leaflet-shaped tissue construct development. To obtain a tissue construct with uniform thickness, a cavity of uniform width was made by using a two-part system mold – a leaflet-shaped core structure and a leaflet-shaped cover (Figure 3A & B) [12]. In the control samples, any scaffold material was not included because in addition to the orientation of cells and their deposited proteins, we investigated the effect of fibers on different aspects of tissue engineering including cell infiltration and spreading, ECM production, phenotypes of cells and other biological and physical properties of tissues. In our previous studies, we performed *in vitro* and *in vivo* tissue engineering with a prototype flat trilayered fibrous scaffold [13,33]. We found that *in vivo* tissue-engineered constructs were more robust and had a distinct trilayered tissue structure compared with *in vitro* tissue-engineered constructs. Thus, in this study, we were interested on *in vivo* tissue engineering of heart valves. During *in vivo* tissue engineering, cells infiltrated into the cavity area and scaffolds present in the cavities were automatically infiltrated by cells. Developed scaffold-based tissue constructs had a diversely oriented, trilayered structure that was not seen in control tissue constructs. The trilayered, oriented fibrous scaffolds were useful to influence the infiltrated cells and their deposited ECM proteins, mainly collagen fibrils to be aligned along the oriented fibers to bring the required structure in the developed tissue constructs. In the FFT analysis on degree of alignment of collagen fibrils, a narrow peak in the fibril alignment plot against angle of acquisition confirmed the alignment of collagen fibrils in the aligned layers of the trilayered tissue constructs. TEM images of the trilayered tissue constructs further confirmed the formation of required orientations of the collagen fibrils. As the scaffolds were made of a biodegradable material, the biomaterial in the constructs would be replaced by ECM including collagen fibrils and complete tissue constructs would be developed over time after heart valve replacement [34].

Degradation of PCL material in the fibrous scaffolds took place during 3 months *in vivo* tissue engineering, and that is why tensile properties of the were less than that of scaffolds and higher than that of control tissue constructs. Tensile moduli of trilayered tissue constructs in circumferential direction (4.173 ± 1.159 MPa) and in radial direction (2.236 ± 0.382 MPa) were more than enough to bear native physiological pressure [35,36]. After heart valve replacement, the remaining scaffold materials in the tissue-engineered leaflet constructs would degrade and tissue growth would occur further resulting in development of complete tissue leaflet constructs with native properties. Cells from blood and aorta wall would infiltrate into the tissue constructs further for their growth [37]. *In situ* dynamic environment would remodel the leaflet constructs into native leaflets.

The scaffolds and trilayered tissue constructs had quite similar anisotropic mechanical characteristics, i.e. during tissue engineering, mechanical characteristics of the PCL scaffolds in both circumferential and radial directions changed proportionately. Thus, if a trilayered fibrous scaffold is made with required anisotropic properties, it is expected that a trilayered tissue construct developed with that scaffold will have the required anisotropic properties. Anisotropic ratios of trilayered tissue constructs (modulus: 1.6 and strength: 2.5) were much lower than that of native leaflets (modulus: 15 and strength: 7). The reason is that TS were made of single polymer (PCL), whereas a native leaflet is made of three main ECM components – collagen, GAG and elastin – and collagen is much stronger than elastin. Thus, to achieve anisotropic properties of a native leaflet in a TS, other biodegradable soft polymers such as polyglycerol sebacate should be included in scaffold fabrication individually or as composites (with PCL) [4,38,39].

Previous research studies have stated that under polarized light, red color collagen fibrils are more mature than the green color collagen fibrils [40,41]. Fibers in the scaffolds might have promoted the maturity of collagen fibrils. Beyond collagen fibrils, GAG and elastin were present in the constructs and were aligned in the respective layers. Presence of aligned fibers in the scaffolds influenced the cells to deposit these protein components in particular orientations. In a native leaflet, these components are layer specific – collagen fibrils in the fibrosa layer, GAG in the spongiosa layer and elastin in the ventricularis layer. However, these ECM components were present quite uniformly

throughout the tissue-engineered constructs. After heart valve replacement, remodeling of tissue constructs would take place in the native dynamic environment and progressively, the constructs would change toward the native structure [36,37]. Quantification study of these ECM materials showed that except for collagen, relative quantities of GAG and elastin in trilayered and control tissue constructs had no significant differences. Although, bright field images of control tissue constructs showed the presence of collagen throughout the constructs, their polarized light images showed the immaturity of the collagen in the constructs. Previous research reports suggest that cellular adhesion to substrates and their interaction with them are affected by substrate morphology and its mechanical properties, which in turn influence the deposition of ECM proteins and their quality [42,43]. Thus, presence of aligned microfibers in the scaffolds could be responsible for the maturity of the collagen in the scaffold-based tissue constructs.

Through protein marker staining, infiltrated cells in the tissue constructs were found to have vimentin and α -SMA expression. While the sole vimentin signal represents quiescent fibroblast phenotype, vimentin and α -SMA signals in cells together represent their myofibroblast phenotype that designates the growing or disease state of the tissues/organs [29,44]. In this case, the scaffolds were placed subcutaneously for *in vivo* tissue engineering of TCs, so the tissue constructs were in growing state (not in diseased state). Similarly, the control tissue constructs were also in growing state. During tissue development, infiltrated cells interacted mainly with TS in test samples whereas they interacted with the mold material (ABS polymer) only, in control samples and mechanical properties of the fibrous scaffolds were much less than that of the mold material. Mechanical properties of the scaffolds and their surrounding mold material influenced the myofibroblast expression by the cells and higher mechanical properties of the mold material in control samples might have caused higher myofibroblast expression [16,28,45]. Thus, both vimentin and α -SMA expression were higher in the control tissue constructs than in the trilayered tissue constructs.

After subcutaneous implantation of the molds with or without scaffolds, inflammation and migration of M1 phenotype macrophage cells into the inflammation zone occurred [22,46]. They were followed by repair and tissue growth steered by migration of M2 phenotype macrophage cells. After 3 months of implantation, both types of macrophage cells were observed in the tissue constructs. In control tissue constructs, presence of both M1 and M2 cells was significantly higher than that in trilayered tissue constructs. However, in trilayered tissue constructs, M1 phenotype signal was low and M2 phenotype signal was comparatively high, in other words, microfibers in the scaffolds could be responsible for this occurrence [23,47]. This occurrence was supported by higher tissue growth in scaffold-based tissue constructs compared with that in control tissue constructs, confirmed by other characteristics discussed before.

During *in vivo* tissue engineering, blood vessels are formed in general; they could be in growing state and they may not be visible clearly. In our previous studies (*in vivo* tissue engineering with flat trilayered fibrous scaffolds), we found almost no endothelial cells (using Von Willebrand factor staining method) in the test samples (with scaffolds) but we found few endothelial cells where there were blood vessels in the control tissue samples (without scaffolds) [13,33]. Similar phenomena can be observed visually in the test and control construct tissue construct samples in this study (Figure 3I & J). Although few blood vessels were observed in the control tissue construct samples, not a single test tissue sample showed any blood vessel.

Conclusion

In this study, trilayered, oriented fibrous scaffolds mimicking the orientations of trilayered native leaflets were developed in an electrospinning system. Their fiber diameter was in microscale. Pore size of the scaffolds was sufficient for easy cell infiltration during tissue engineering. After 3-month *in vivo* tissue engineering, infiltrated cells and their deposited ECM components were found aligned in respective layers of the tissue-engineered constructs forming trilayered tissue constructs. Tensile properties of the trilayered tissue constructs were sufficient to bear physiological load. Expression by the infiltrated cells in the tissue constructs confirmed tissue growth status of the constructs. Presence of higher number of M2 phenotype macrophage cells compared with M1 phenotype macrophage cells in the trilayered tissue constructs also demonstrated that fibers in the scaffolds influenced tissue growth positively.

Translational perspective

Heart valve tissue engineering has the potential to solve the problems caused by valvular heart diseases. A heart valve, especially each leaflet, has a complicated structure and bears a complex dynamic load in three modes – tension, shear and flexure. This study concentrated on development of autologous leaflet-shaped constructs mimicking

the structure of native leaflets. To make it successful, microfiber-based scaffolds were developed to invoke positive cells during *in vivo* tissue engineering considering the translational perspective. In the tissue constructs, residing fibroblast-type cells were sensitive to the structure of the constructs and it is expected that native-mimicked leaflet-shaped tissue structure would bring positive outcomes in long-term. Our ultimate goal is to develop an autologous functional heart valve and our future work will be engaged with the development of a heart valve scaffold mimicking the structure and morphologies of a native heart valve. Autologous heart valves will then be developed through *in vivo* tissue engineering using the fabricated heart valve scaffolds and the developed autologous valve tissues could be useful for heart valve replacement.

Summary points

- This study focused on development of autologous leaflet-shaped tissue constructs mimicking the morphology of native heart valve leaflets.
- Trilayered microfibrous scaffolds mimicking the structure of native heart valve leaflets were prepared.
- The scaffolds were implanted in a rat model subcutaneously for *in vivo* tissue engineering.
- Tensile properties of the leaflet tissue constructs were sufficient to bear physiological load.
- Infiltrated cells and their deposited extracellular matrix components including collagen, glycosaminoglycans and elastin were oriented in respective layers, thus formed trilayered tissue constructs.
- Collagen fibrils in the constructs were matured due to presence of fibers in the scaffolds.
- Presence of fibers in the scaffolds lowered the myofibroblast expression by the infiltrated cells.
- Microfibers in the scaffolds made positive environment for migration of growth-oriented M2 phenotype macrophage cells.
- Native-mimicked, trilayered and oriented microfibrous scaffolds influenced *in vivo* tissue engineering during the development of autologous leaflet-shaped tissue constructs.

Author contributions

S Jana designed and performed the study, analyzed the results and wrote the manuscript. A Lerman supervised the study.

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Ethical conduct of research

The authors state that they have obtained approval of the Ethical Committee of Mayo Clinic, Rochester, MN, USA to perform an *in vivo* tissue engineering study in a rat model.

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