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A novel surgical technique for a rat subcutaneous implantation of a tissue engineered scaffold

Reza Khorramirouz, Jason L. Go, Christopher Noble, Soumen Jana, Eva Maxson, Amir Lerman, Melissa D. Young*

Department of Cardiovascular Medicine, Mayo Clinic, Rochester, MN, United States

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ABSTRACT

Objectives: Subcutaneous implantations in small animal models are currently required for preclinical studies of acellular tissue to evaluate biocompatibility, including host recellularization and immunogenic reactivity.
Methods: Three rat subcutaneous implantation methods were evaluated in six Sprague Dawley rats. An acellular xenograft made from porcine pericardium was used as the tissue-scaffold. Three implantation methods were performed; 1) Suture method is where a tissue-scaffold was implanted by suturing its border to the external oblique muscle, 2) Control method is where a tissue-scaffold was implanted without any suturing or support, 3) Frame method is where a tissue-scaffold was attached to a circular frame composed of polycaprolactone (PCL) biomaterial and placed subcutaneously. After 1 and 4 weeks, tissue-scaffolds were explanted and evaluated by hematoxylin and eosin (H&E), Masson's trichrome,Picrosirius Red, transmission electron microscopy (TEM), immunohistochemistry, and mechanical testing.
Results: Macroscopically, tissue-scaffold degradation with the suture method and tissue-scaffold folding with the control method were observed after 4 weeks. In comparison, the frame method demonstrated intact tissue scaffolds after 4 weeks. H&E staining showed progressive cell repopulation over the course of the experiment in all groups with acute and chronic inflammation observed in suture and control methods throughout the duration of the study. Immunohistochemistry quantification of CD3, CD 31, CD 34, CD 163, and αSMA showed a statistically significant differences between the suture, control and frame methods ($P < 0.05$) at both time points. The average tensile strength was 4.03 ± 0.49, 7.45 ± 0.49 and 5.72 ± 1.34 (MPa) after 1 week and 0.55 ± 0.26, 0.12 ± 0.03 and 0.41 ± 0.32 (MPa) after 4 weeks in the suture, control, and frame methods; respectively. TEM analysis showed an increase in inflammatory cells in both suture and control methods following implantation.
Conclusion: Rat subcutaneous implantation with the frame method was performed with success and ease. The surgical approach used for the frame technique was found to be the best methodology for *in vivo* evaluation of tissue engineered acellular scaffolds, where the frame method did not compromise mechanical strength, but it reduced inflammation significantly.

1. Introduction

Certain aspects of tissue response to biomaterials are important from research and development perspectives.The *in vivo* evaluation of tissue reaction to these materials is important for performance, safety, and regulatory reasons. The International Organization of Standardization and the Food and Drug Administration (FDA) indicate the required tests needed for the introduction of different biocompatible tissues (Anderson, 2001). During the early stages of development, *in vivo* evaluation provides researchers insight to the proposed manufacturing processes and design of tissue engineered scaffolds. Additionally, these tests should be repeated with the final manufacturing and sterilization conditions found in the final product.

Currently, subcutaneous implantation in small animal models are used for preclinical testing to evaluate immune reactivity and recellularization capacity (Neethling et al., 2010; Rennert et al., 2013; Sarkanen et al., 2012). Although it is common to use different animals such as rabbits and mice for subcutaneous implantation; rat subcutaneous implants are one of the most studied because it enables the assessment of tissue compatibility such as possible sensitization, irritation, intracutanous reactivity, systemic toxicity, genotoxicity, implantation, chronic toxicity, carcinogenicity, biodegradation, and

* Corresponding author at: 200 First St SW, Rochester, MN 55905, United States.
E-mail address: Young.Melissa@mayo.edu (M.D. Young).

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immune response (Modulevsky et al., 2016; Wang et al., 2017; Cunniffe et al., 2015; Ripamonti, 1996). Despite time and costs, it is crucial to have a reliable method for implantation. Subcutaneous implantation allows for the observation of an inflammatory response, cellularization of the extracellular matrix, and angiogenesis validation (Modulevsky et al., 2016; Guarnieri et al., 2014; Stapleton et al., 2010; Meagher et al., 2016; Mazza et al., 2015).

Various scaffolds have been explored with a subcutaneous model of implantation such as Polylactides (PLA), poly(3-hydroxybutyrate), porcine small intestine submucosa, and bioengineered acellular scaffolds (Gogolewski et al., 1993; Zheng et al., 2005; Yang et al., 2008; Nillesen et al., 2007). Most studies use the same technique for subcutaneous implantation by placing the biomaterial under the skin without fixation (Kim et al., 2007). The current study suggests that the method of subcutaneous implantation could affect recellularization and inflammatory outcomes (Setzen and Williams, 1997). Therefore, it is crucial to have a stringent methodology that will minimize tissue degradation in the host, while minimizing inflammation and maximizing the recellularization capacity. We hypothesize that application of a PCL composed frame as a support for an acellular scaffold will provide better outcomes in the subcutaneous location of a rat reducing inflammation and better biomechanical properties.

2. Materials and methods

2.1. Procurement, decellularization, and sterilization of porcine pericardium

Freshly harvested porcine pericardium was obtained from a local abattoir (Hormel Food Corporation, USA). The tissue was stripped of adjacent fat and rinsed with phosphate buffered saline solution (PBS). The tissue was processed with sodium dodecyl sulfate (SDS), DNase, and diH_2O with constant agitation for 2 days at room temperature. An additional exposure and wash with DNase, Tris buffer, $MgCl_2$, peroxyacetic acid (PAA), and phosphate buffered solution (PBS) was performed. The decellularized pericardial tissue was sterilized using supercritical carbon dioxide (NovaSterilis, Inc., USA). The detailed decellularization and sterilization process parameters are outlined in our previous work (Hennessy et al., 2017).

2.2. Biomaterial frame composition

The frame-like structures used to support the implanted tissue were 3D-printed using an Envision TEC 3D-Bioplotter. The 3D computer aided drawing (CAD) model of the ring was generated, with an inner and outer diameter of 19.2 mm and 22.7 mm, respectively. The CAD model was uploaded to the Magics Envision TEC software (32 bit, version 16.2.0.20, Materialize n.v. 2011) where it was modified before being uploaded to the Bioplotter software (version 3.0.713.1406, Envision TEC), which enables slicing of the model, before 3D-printing. The slice thickness utilized was 320 μm. The resulting file was uploaded to the software Visual Machines (version 2.8.126) that allows the user to input the various parameters that control the bioprinter. Polycaprolactone (PCL, Mn 45,000, and Sigma-Aldrich) beads were loaded into the high temperature stainless-steel cartridge and heated to 130 °C for 2 h. The rings were printed onto an inverted 120 mm diameter glass petri dish (Duroplan, Germany) that was stabilized by a petri dish holder. Nine rings were printed at a time, side-by-side, each made of one extruded layer of PCL. The strands of PCL were printed 0.4 mm apart, with a needle vertical offset of 0.05 mm and using a stainless steel needle of 0.7 mm inner diameter. The printing platform temperature was kept at 35 °C, and the cartridge at 130 °C throughout the printing process, the speed and extrusion pressure were set to be 2.3 mm/s and 6 bar, respectively. Reproducibility of the PCL support frames was ensured by using the same CAD model for each frame, and by the high XYZ axis resolution of the Bioplotter (0.001 mm).

2.3. Surgical implantation

Six Sprague Dawley rats were obtained and were raised in a pathogen-free environment throughout the experiment. All experiments were performed in strict accordance with the recommendations in the Guide for Institutional Animal Care and Use Committee (protocol number: A00001864-16). The rats were classified into 3 groups with different surgical implantation methods, 2 rats for each method produced a total of 8 specimens per group. The rats were induced by isoflurane 4% and maintenance of 2% and shaved, and then the buprenorphine (0.6 mg/kg) was injected subcutaneously. The animals were positioned in prone to provide better oxygenation. After scrubbing with betadine, the 20 mm dorsal midline incision was made over the thoracolumbar area. Two tissue scaffolds were inserted at both sides. The adjacent fascia was released and three surgical methods were applied:

Method 1 (suture): The acellular porcine pericardium was cut into 20 mm × 20 mm segments and placed over the muscle under the skin and the scaffold was sutured at four corners with Polypropylene 5-0.

Method 2 (control): The scaffold was cut the same as above and placed subcutaneously without suturing and fixation.

Method 3 (frame): The scaffold was fixed to the PCL rim frame by Polypropylene 5-0 under sterile conditions. Then the fixed scaffold was placed between skin and fascia and left in position.

All the animals were closed with Vicryl 4-0 and received topical tetracycline and followed-up for 1 and 4 weeks post-implantation.

2.4. Histology and immunohistochemistry

After 1 and 4 weeks, tissue samples were biopsied, sectioned, and paraffin-embedded. The samples underwent Hematoxylin-Eosin (H&E), Masson's trichrome, and Picrosirius red staining per manufacturer's protocol. Recellularization and immune reaction were evaluated using immunohistochemistry with various antibodies. Recellularization of endothelial-like and interstitial-like cells was evaluated using CD 34 (Abcam, Cambridge, UK), CD 31(Abbiotec, San Diego, CA), alpha smooth muscle actin (Sigma, St. Louis, MO), and vimentin (Abcam, Cambridge, UK) while immune reaction was evaluated using CD 168 (Abcam, Cambridge, UK) and CD 3 (Abcam, Cambridge, UK) antibodies. Tissue samples were labeled with the Dako Envision System-HRP, blocked with the Dako peroxidase solution incubated in primary antibody overnight at 4 °C with the secondary antibody (biotinylated rabbit anti-rabbit Ig F [ab'] 2 fragments; Dako). All the images were captured using 10× and 20× magnification by light microscopy. All images were analyzed using Image J software, which was previously determined to be a validated method (Vogel et al., 2016). Quantitative assessment of Picrosirius staining was done by capturing 8 images in different regions across the tissue sample. The stained area was measured and presented as percent area. For DAPI staining the same procedure was done and cell signaling was counted per area and reported as percent area. For immunohistochemistry the positive stained area was measured.

2.5. Transmission electron microscopy

Tissues were fixed in Trumps fixative at 4 °C overnight per manufacturer's protocol. These were then washed in PBS, rinsed in water, fixed in 1% osmium tetroxide (OsO4), dehydrated through a graded series of ethanol, and embedded in Spur resin. Ultra-thin sections (100 nm or 0.1 μm) were cut and mounted on 200-mesh copper grids. After being post-stained with lead citrate, they were imaged in a JEOL (JEM-1400 Plus) transmission electron microscope.

2.6. Biomechanical properties: uniaxial mechanical testing

The biomechanical properties were evaluated at 1 and 4 weeks for all 3 methods. The testing was conducted on 4 samples with each group,