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Full Thickness Heart Repair with an Engineered Multilayered Myocardial Patch in Rat Model

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Abstract

Structural cardiac defects, such as Tetralogy of Fallot, often requires surgical placement of a patch to expand the right ventricular outflow tract (RVOT) in an area normally consisting of contractile myocardial tissue. Current cardiac patch materials are biologically inert and will not grow with a pediatric patient, often requiring reoperations. In this study, novel multi-layered scaffolds with a polycaprolactone core, a chitosan-based scaffold, and either gelatin or decellularized porcine heart matrix were implanted into a full thickness rat right ventricle defect for up to 8 weeks. The results show that engineered scaffolds were biodegradable and promoted tissue remodeling. Histological analysis of control fixed pericardium patches showed little to no cellular infiltration, while engineered scaffolds had significant muscular and vascular cell remodeling. Quantitative MRI revealed that left ventricular ejection fractions were stabilized in all patched hearts after 8 weeks, and the right ventricular ejection fraction in hearts with engineered patches was significantly greater than hearts with control pericardium patches. In addition, patches with heart matrix promoted a denser vascular network and a higher M2/M1 inflammatory macrophage ratio when compared to patches containing only gelatin. Collectively, these results show that these multi-layered patches are capable of full thickness defect repair and regeneration.

Keywords

heart patch; congenital heart defects; chitosan; polycaprolactone; right ventricular outflow tract

1. Introduction

Tetralogy of Fallot (ToF) affects 3 to 6 of every 1000 live births in the U.S. and usually necessitates surgical placement of a patch across the right ventricular outflow tract (RVOT) that consists of conductive and contractile myocardial tissue.^[1] Current patches consist of glutaraldehyde-cross-linked bovine pericardium, woven nylon, expanded polytetrafluoroethylene and porcine small intestinal submucosa. These materials are highly

successful in the short-term. However these materials are not biodegradable and cannot grow with a pediatric patient's heart and are prone to calcification, necessitating replacement in about 14% of patients.^[2] In addition, non-biodegradable and non-regenerable patches induce an inflammatory foreign-body response, resulting in encasement of the material within a fibrous scar-like tissue that lacks elasticity and hinders the restoration of regional tissue functionality.^[3] As a result, patients with these heart patches have an increased risk of infection, heart failure, arrhythmia and aneurysm.

The ideal cardiac patch would consist of materials that combine biocompatibility, biodegradability and sufficient tensile strength with elasticity similar to native cardiac tissue.^[5] Furthermore, future cardiac patches should also have the ability to regulate cellular migration, repopulation and growth. Previously, we reported a novel process of generating a self-assembled polycaprolactone (PCL) core in an aqueous medium, which decreased hydrophobic surface properties while maintaining high tensile strength.^[6] We also showed that a multi-layered scaffold with a gelatin/chitosan hydrogel surrounding this PCL polymer core was biodegradable, has sufficient mechanical strength, and maintained cardiomyocyte viability for cardiac patch applications. We further demonstrated that replacing gelatin with decellularized porcine heart extracellular matrix in this multi-layered scaffold resulted in improved contractile and electrophysiological functions in an *in vitro* neonatal rat ventricular myocyte model.^[7] In this study, we investigated this multi-layered patch system in a rat model to assess the feasibility and efficacy for full thickness reconstruction of the RVOT. In our previous paper,^[7] we formulated the mechanical properties of the gelatin and cECM patch to be identical. Heart matrix/chitosan blended hydrogels (1.6 mg/mL heart matrix) had similar porosity (109-34 mm), and elastic modulus (13.2-4.0 kPa) as gelatin/chitosan scaffolds. We compared this patch to commercially available fixed bovine pericardium control (SJM™, MN, U.S.).^[7, 8] We hypothesized that both novel multi-functional patches would provide suitable mechanical properties, biocompatibility, and biodegradability, and that heart extracellular matrix based patch would improve cell immigration and growth *in vivo* compared to commercially available fixed bovine pericardium.

2. Results and Discussion

Gelatin or heart extracellular matrix based patches (6 mm in diameter and 3 mm in thickness) and fixed bovine pericardium patches (6 mm in diameter and 0.4 mm in thickness) were sutured into the RVOT free wall of adult Sprague Dawley rats following the creation of a full thickness defect (Figure 1A). All patches provided surgical handling and adequate suture holding strength without bleeding through the patched area (Figure 1B(1,4,7)). All patches were incorporated into the native tissue at 4 weeks of post-surgery (Figure 1B(2,5,8)). Surfaces of the right ventricle (RV) were covered by layers of scar tissue as a part of the remodelling process. At 8 weeks post surgery, most of these scarfibers had disappeared. Furthermore, both gelatin and heart extracellular matrix patches showed a reddish and a yellowish appearance on the patched area (Figure 1B(6,9)) after 8 weeks, whereas pericardium patches showed only a yellowish appearance (Figure 1B(3)). This suggested that hydrogel based patches promoted vascular tissue formation through the patched area whereas the pericardium patch showed a lack of cellular infiltration and possible fibrosis without neovascularization. Both multi-layered patches were intentionally

manufactured to be 4-mm thick in order to encourage tissue regeneration while current commercial patches, used as controls, were much thinner (<1mm). Thus, multi-layered patches initially protruded from the RV slightly. The thicknesses of both gelatin and heart extracellular matrix patches reduced significantly over time as patch materials degraded and tissue regeneration progressed (Figure 1C). At 4 weeks post surgery, gelatin patches were significantly thicker than heart extracellular matrix patches ($P<0.05$). However, after 8 weeks, the thickness of both engineered patches was equivalent to the native heart wall thickness. This is likely due to the faster degradation rate of heart extracellular matrix patches compared to gelatin patches. Furthermore, the thickness of the PCL core reduced significantly over time (Figure 1D). The thickness of the PCL core in both engineered samples decreased by 40% after 8 weeks ($P<0.05$ vs Day 0), where as *in vitro* degradation of the same PCL core had a 10% weight loss after 50 days, suggesting that *in vivo* degradation of the self-assembled PCL core was faster than *in vitro*. Previous studies also reported that PCL scaffolds degraded faster *in vivo* as compared to that *in vitro* due to enzymatic degradation in addition to the hydrolytic one.^[9]

The multifunctional design of the engineered patches provides mechanical strength and elasticity during the tissue regeneration process. To evaluate the effects of patches on cardiac function, blood volumes of left and right ventricles at end-diastole and end-systole were measured at 2, 4 and 8 weeks post surgery using MRI (Figure 2A). No samples displayed RVOT obstruction during MRI analysis (figure not shown). End-diastolic volumes (EDV) in both LV and RV increased significantly after 8 weeks (Figure 2C and D) followed by heart weight increases over time, particularly in male rats (Figure 2B). However, the end-systolic volumes (ESV) in the LV and RV showed no significant change (Figure 2E and F). Left and right ventricular ejection fractions were similar to previously reported rat models.^[10,11] However, there was high variability in measuring right ventricular ejection fraction (RVEF) due to the non-uniform shape of right ventricles, particularly when excluding papillary muscles. Our results showed, compared to non-surgery, LVEF of all patched hearts were significantly reduced 2 weeks after surgery ($P<0.05$) and then normalized after 4 weeks (Figure 2G). However, RVEF was significantly lower in the pericardial patch compared to no surgery at 8 weeks but the engineered materials were not significantly different when compared to non-surgery ($P<0.05$) (Figure 2H). Such differences may be the result from variations in stiffness between pericardium and the PCL core in the engineered patches. Both pericardium and PCL are viscoelastic materials; but the elastic modulus of pericardium (~80 MPa in transversal and ~70 MPa in root-to-apex^[12]) is significantly higher than the elastic modulus of the PCL core (~300 kPa, isotropic^[8]).

M1 and M2 macrophages, resulting from classic and alternative activation, play important roles in the host inflammatory response and the process of tissue regeneration following device implantation or injury. M1 macrophages are comprised of immune effect or cells with an acute inflammatory phenotype that are highly aggressive against bacteria and produce large amounts of lymphokines. M2 macrophages can induce signaling molecules such as fibroblast growth factor, vascular endothelial growth factor, immunoregulatory factors (e.g. IL10 and TGF- β 1) and extracellular matrix remodeling factors (e.g. FGF1 and MMPs).^[13] Based on these studies, H&E staining and expression markers of CD86 (M1) and CD206 (M2) were used to investigate M1 and M2 macrophage phenotype localization on patched

areas at 4 weeks after surgery. H&E staining of sectioned patches showed that immune responses were evenly distributed throughout both engineered patches (Figure 3A(2,3)), whereas immune responses were only found on the surface of the pericardium patches (Figure 3 A(1)). Immunofluorescence staining demonstrated that macrophages actively participated in the remodeling of engineered patches, including i) positive staining for CD86 suggesting the presence of M1 macrophages which are newly recruited inflammatory cells (Figure 3D(1,4)) and ii) positive staining for CD206 suggesting the presence of M2 macrophages that facilitate reconstruction (Figure 3D(2,5)). Note that several cells are co-labeled with both markers CD86 and CD206. Mantovani and colleagues proposed an M1-M2 macrophage model, in which M1 included IFN γ +LPS or TNF produced by T_H1 cells, and M2 was subdivided to accommodate similarities and differences between IL-4 (M2a), immune complex + Toll-like receptor (TLR) ligands (M2b), and IL-10 and glucocorticoids (M2c) produced by T_H2 cells. M2b macrophages express CD86 but M2a and M2c macrophages do not; M2c macrophages are responsible for matrix deposition and tissue remodeling as well as for immunoregulation. The above-mentioned model can help explain why in Figure 3D(6) some cells were co-labeled with CD86 and CD206 and some were labeled with CD86 and CD206 separately. In addition, the numbers of macrophages were markedly increased after 4 weeks within the area of engineered patches but without significant difference between gelatin and heart extracellular matrix patches (Figure 3B). However, heart extracellular matrix patches showed significantly higher M2/M1 ratios than gelatin patches ($P<0.05$) (Figure 3C) suggesting faster constructive remodeling compared to gelatin based patches due to the increased presence of the alternative activation M2 macrophages.

Cardiac tissue replacement by surgical patching is limited by the inability to grow and remodel vascular networks. An ideal biodegradable patch should allow nutrients and waste to diffuse throughout the patch for cell growth, migration and population. After 8 weeks post surgery, H&E staining indicated that there was no calcification in all samples (Figure 4A (7-9)). Pericardium patches showed yellowish appearance indicating a lack of cellular infiltration or possible further fibrosis (Figure 4A (1)) and no significant cell invasion into the patch (Figure 4A (7)). However, engineered patches showed a reddish appearance. Microscopic views showed blood vessel formation within the hydrogel region of engineered patches (Figure 4A(5,6)). H&E staining confirmed that endothelial cells invaded into engineered patches (Figure 4A(8,9)) and had positive expression of α -smooth muscle actin and CD31 (Figure 4D(1,3)). Quantitatively, endothelial cell density of engineered patches was significantly higher (Figure 4B) and invaded further (Figure 4C) than those in the pericardium patch ($P<0.05$), and compared to gelatin patches endothelial cell density is even higher ($P<0.05$). Also, endothelial cell density showed large variability depending on the specific patch regions. Even though endothelial cells invaded into the middle of hydrogels, cell density was reduced significantly in the middle of hydrogels. Furthermore, muscular tissue formed and aligned surround the PCL core with positive staining for cardiac troponin T (cTnT) and Connexin 43 (Cx43) (Figure 4D(2,4)). There was no significant difference in the thickness of muscular tissue between gelatin and heart extracellular matrix based patches.

One of the major concerns in current cardiac patch implantation is the encasement of the material within fibrous scar-like tissue. Scarring fibrosis replacement, mainly type 1 collagen, is formed by fibroblast accumulation and excess deposition of extracellular matrix (ECM) proteins causing increased stiffness, pathological signalling and impaired electromechanical coupling of cardiomyocytes.^[14] In order to understand the behaviour of a composite material implanted in the rat heart, rats were sacrificed at 4 and 8 weeks of implantation. Heart sections were stained for Masson's Trichrome indicating the presence of collagen which is a major connective tissue protein produced and released by fibroblasts. Our analysis showed highly collagenous areas in all samples due to dense inflammatory connective tissue formation after 4 weeks of implantation (Figure 5A(1-3)). Particularly, more collagen was found surrounding the PCL core and reconstructed area of the right ventricle wall side (Figure 5A(5,6)).

Quantified analysis of sectioned tissue images demonstrated that collagenous area on both engineered patches reduced significantly by 8 weeks compared to that at 4 weeks ($P<0.05$), whereas pericardium patches no significant change in collagenous area (Figure 5). This is probably due to cell invasion and growth in engineered patches resulting in an active constructive remodelling process.

In conclusion, these results demonstrate that multi-layered engineered patches are remodelled by both muscular and endothelial tissue while pericardium does not promote cell invasion. Engineered patches maintain higher right ventricular ejection fractions compared to commercially available fixed bovine pericardium at 8 weeks post surgery. In addition, heart extracellular matrix patches promote a denser vascular network and a higher M2/M1 inflammatory macrophage ratio. Future research will involve quantifying fibrotic response at PCL core, populating patches with cardiomyocytes and endothelial cells, and implanting the min a large animal model.

3. Experimental Section

Patch preparation

Multi-layered patches with PCL cores and chitosan-heart extracellular matrix hydrogels were made using previously published procedures.^[6, 8] Briefly, 10% (w/v) PCL solutions with equal parts of 10 kDa and 80 kDa in glacial acetic acid solution was pipetted into a custom-made Teflon mold containing 2 mL of water and self-assembly formed solid matrices (diameter = 6 mm). Formed PCL cores were sandwiched in a blend of 2% (w/v) gelatin/2% (w/v) chitosan or 0.5% (w/v) heart extracellular matrix/1.5% (w/v) chitosan composite hydrogels and lyophilized at -50°C for 24 hours. Formed scaffolds were neutralized using 100% ethanol and rehydrated using PBS. A commercially available fixed bovine pericardium (SJM™, MN, U.S.) was used as a control.

Surgical procedure

All studies involving experimental animals were approved by the Institutional Animal Care and Use Committees of both Rice University and Baylor College of Medicine. Six month old Sprague-Dawley rats (200 ~ 300 g body weight) of both sexes were used for full

thickness RVOT repair surgery using previously published procedures with modifications. [10] Rats were anesthetized using 4% isoflurane, endotracheally intubated and mechanically ventilated with a small animal respirator (Harvard Co.) at a frequency of 70 – 75 breaths/min and tidal volume of 1.5 – 2.5 ml depending on the body weight of the rat. Rats were kept anesthetized with 100% O₂ and 1.25 – 1.5% isoflurane. Body temperature, heart rate, respiratory rate, and arterial oxygen saturation was monitored using an EKG monitoring system. The rat heart was exposed through a median sternotomy under using aseptic techniques with sterile instruments. A purse-string suture (diameter of 4 mm) was placed in the free wall of the RV with 7-0 polypropylene sutures. Both ends of the stitch were passed through a 22-gauge plastic vascular cannula as a tourniquet. The bulging part of the RV wall inside the purse-string stitch was resected. A transmural defect was confirmed by briefly releasing purse-string stitch. A patch was sutured along the margin of the purse-string suture with over-and-over sutures with 7-0 polypropylene to cover the hole in the RV. The sternum was closed parasternally with four interrupted sutures of 2-0 polypropylene after the expansion of lungs using positive end-expiratory pressure. The muscle layer was closed with 4-0 polyglactin absorbable sutures, and the skin layer was closed with a tissue stapler. Isoflurane supply was stopped immediately after closing the skin layer. Buprenorphine (0.5mg/kg) was administered intraperitoneally twice/day for 3 days for post-surgical pain relief.

MRI imaging

All rats were imaged using MRI at 2, 4 and 8 weeks after surgery to measure cardiac function. All images were acquired with a 9.4T, BrukerAvance III Biospec Spectrometer, 21 cm bore horizontal scanner with a 35mm volume resonator (BrukerBiospin, Billerica, MA). Anatomical images were acquired using the retrospective gating Paravision 5.1 software package (Intragate) to acquire cine cardiac MRI movies throughout the cardiac cycle. Ventricular Volumes were analyzed using Amira® software or Cmr42.

Histology

Rats were euthanized at 4 and 8 weeks post surgery, fixed in 10% formalin, dehydrated and embedded in paraffin for sectioning as described previously with minor modification. Short axial sections were stained with haematoxylin and eosin (H&E; Leica Biosystems, Richmond, IL) and Masson's Trichrome (Sigma Aldrich, St. Louis, MO). Immunofluorescence staining of CD86 (1:100; ab53004, Abcam), CD206 (1:200; ab64693, Abcam), α -smooth muscle actin (α -SMA; 1:50; ab7817, Abcam), CD31 (1:50; ab28364, Abcam), cardiac troponin T (cTnT; 1:100; ab8295, Abcam) and connexin 43 (Cx43; 1:100; ab11370, Abcam) were used to reveal macrophage participation, density of blood vessels and distribution of electromechanical conjunction in the implants respectively. Slides were counterstained with DAPI (Invitrogen, Eugene, OR). Images were taken using a Nikon-Elements E800 microscope. Data were analyzed using ImageJ software.

Statistics

Values at all conditions and time points are presented as mean $\pm$ SD. Comparisons between groups were made with a student t-test and comparisons among groups were made with a

one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. In all tests, differences were considered statistically significant at $P < 0.05$.

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Figure 1.

In vivo applications of cardiac patches maintain structural integrity and promote tissue remodeling. A, Schematic of how cardiac patches were applied to repair full thickness RVOT defects. (1) Purse string suture applied over RVOT. (2) Sutures are gathered to form tissue pocket and defect is excised from tissue pocket. (3) Patch is sutured around purse string defect. (4) Purse string is removed and final closure of patch seals the defect. B, Digital images of patched defects immediately following surgery (Day 0), and after 4 or 8 weeks post surgery. Fixed bovine pericardium control patch (1-3), Gelatin (4-6) and heart extracellular matrix (7-9) based patches with PCL core. Bar=10 mm. C, Total cardiac patch thickness over time. D, Thickness of PCL core in gelatin and heart extracellular matrix based patches over time. Data are shown as mean $\pm$ SD (n=5 in each group); * P < 0.05 vs Heart Matrix in C, and * P <0.05 vs Day 0 in D.

Figure 2.

Patch effects on cardiac function through quantitative MRI analysis at 2, 4 and 8 weeks after surgery. A, Representative MRI images of cardiac ventricular end-diastole (ED) and end-systole (ES) from patched hearts. B, Heart weight over time comparing male to female rats. C and D, LV and RV end-diastolic volumes (EDV) over time. E and F, LVESV and RVESV over time. G and H, LVEF and RVEF percentage changes in patched and non-patched hearts over time. Data are shown as mean $\pm$ SD (n=5 in each group); * P <0.05 vs female in B and * P <0.05 vs non-surgery in G and H.

Figure 3. Histological analysis of patched cardiac tissue areas at 4 weeks post surgery show host inflammatory responses. A, H&E stained sections show undegraded fixed pericardium (1) in the control patch, remaining PCL in gelatin patches (2) and in heart extracellular matrix patches (3). Bars=100 μ m. B, Number of macrophages in gelatin and heart extracellular matrix patches. C, Comparison of M2/M1 ratios between gelatin and heart extracellular matrix patches. D, Immunohistochemistry staining of gelatin and heart extracellular matrix patched areas. Positive staining for CD86 and CD206 are indicative of M1 (1,4) and M2 (2,5) macrophage presence, respectively. Bars=100 μ m. Data are shown as mean $\pm$ SD (n=5 in each group); * P <0.05 vs heart matrix.

Figure 4. Vascular cell recruitment and remodeling of cardiac patched areas at 8 weeks post surgery. A, Representative images of patched areas on hearts show distinct differences in color and vessel formation comparing pericardium (1,4) to gelatin (2,5) and heart extracellular matrix (3,6) patches. H&E stained sections (7-9) of patched areas show no calcification. Arrows indicate endothelial cell migration into patched areas. B, Quantitatively comparing the differences in cell density and depth of cell invasion (C) between cardiac patch groups. D, Immunohistochemistry staining of α -smooth muscle actin (α -SMA) and CD31 (1,3), and cardiac troponin T (cTnT) and connexin 43 (Cx43) (2,4) within the center of the defect region and patch in gelatin and heart extracellular matrix patched areas. Data are shown as mean $\pm$ SD (n=5 in each group); * P < 0.05 vs pericardium in B and C, # P < 0.05 vs Gelatin in B.

Figure 5. Masson's trichrome histological analysis of fibrous tissue formation. Stained patched sections after (A) 4 and 8 weeks. Differences in collagen (blue) content are evident over time between patch groups. Bars=200 μ m for (1)-(3) and Bars=50 μ m for (4)-(12). B, Percentage of fibrous scar tissue at 4 and 8 weeks after surgery for each cardiac patch group. Data are shown as mean $\pm$ SD (n=5 in each group); * P <0.05 vs 4 weeks.