In Vivo Skeletal Muscle Biocompatibility of Composite, Coaxial Electrospun, and Microfibrous Scaffolds

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One weakness with currently researched skeletal muscle tissue replacement is the lack of contraction and relaxation during the regenerative process. A biocompatible scaffold that can act similar to the muscle would be a pivotal innovation. Coaxial electrospun scaffolds, capable of movement with electrical stimulation, were created using poly(ϵ -caprolactone) (PCL), multiwalled carbon nanotubes (MWCNT), and a (83/17 or 40/60) poly(acrylic acid)/poly(vinyl alcohol) (PAA/PVA) hydrogel. The two scaffolds were implanted into Sprague-Dawley rat vastus lateralis muscle and compared with a phosphate-buffered saline injection sham surgery and an unoperated control. No complications or adverse effects were observed. Rats were sacrificed on days 7, 14, 21, and 28 postimplantation and biocompatibility assessed using enzymatic activity, fibrosis formation, inflammation, scaffold cellular infiltration, and neovascularization. Serum creatine kinase and lactate dehydrogenase levels were significantly higher in scaffold-implanted rats compared with the control on day 7, but returned to baseline by day 14. Day 7 scaffolds showed significant inflammation and fibrosis that decreased over time. Fibroblasts infiltrated the scaffolds early, but decreased with time, while myogenic cell numbers increased. Neovascularization of both scaffolds occurred as early as day 7. We conclude that the PCL-MWCNT-PAA/PVA scaffolds are biocompatible and suitable for muscle regeneration as myogenic cell growth was supported.

Introduction

SKELETAL MUSCLE MAKES up 40% of the muscle in the human body and consists of multiple fibers.¹ The unidirectional orientation of muscle fibers creates a large contractile force, which leads to bodily movement.² After skeletal muscle injury, the body's repair process facilitates scar tissue formation, which is followed by decreased functionality.² Recently, muscle cell injections, natural scaffolds, and synthetic scaffolds have all been investigated to regenerate skeletal muscle.^{3–11} Boldrin *et al.* compared direct injection of human myogenic precursor cells (hMPC) to hMPC cellularized poly-lactic-co-glycolide (PLGA) scaffolds. At 4 weeks, the injection site still had a large amount of connective tissue, but the PLGA-implanted site had centrally nucleated, regenerating myofibers. They concluded that scaffold implantation was more efficient for tissue regeneration than direct cell injection.⁵

One of the problems with skeletal muscle tissue engineering is the lack of tissue utility while the area is re-

generating. A possible solution is to use an ionic polymer-metal composite (IPMC) or artificial muscle scaffold. These are comprised of an inner polymer layer with metal plated on either side that can actuate. An IPMC placed in an ionic solution will bend and relax due to ion exchange once an electric field is applied.¹² Our laboratory has already successfully created a microfibrous reverse IPMC, as the inner core contains the synthetic polymer/conductive element and the outer sheath is an ionic polymer gel (IPG).^{13–15}

IPGs are hydrogels that are affected by changes in temperature, pH, light, pressure, ionic strength, and electric field.^{12,16–18} When a pH-sensitive IPG is placed in a salt solution between two electrodes, the anode produces oxygen and the cathode produces hydrogen. This decrease in pH at the cathode and increase at the anode causes the IPG to shrink, expand, or bend.^{12,16} A polyacrylic acid (PAA) and polyvinyl alcohol (PVA) IPG hydrogel is pH sensitive as it shrinks in an acidic solution and expands in a basic one.^{19,20} These two responses can also be achieved using electrical stimulation as discovered by Hamlen *et al.*, but the change

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in size took over 10 min.¹⁹ Caldwell *et al.* studied the rate of actuation based on the thickness of the PAA/PVA fiber and determined that as the thickness was reduced the actuation rate significantly increased.²¹

The unique reverse IPMC scaffold uses coaxial electrospinning to fabricate each scaffold so that individual fibers have an inner polymer core surrounded by a separate polymer sheath. The inner core contains poly(ϵ -caprolactone) (PCL) and acid functionalized multiwalled carbon nanotubes (MWCNT). MWCNT are the conductive element as the hydrogen molecule has been removed from each carbon in the hexagonal lattice and allows free movement of the remaining electrons.^{22,23} The exterior sheath was composed of the PAA/PVA hydrogel. The PCL-MWCNT-PAA/PVA scaffold was successfully coaxial electrospun and it was determined that all three components were necessary for actuation. These scaffolds have also tested well for biocompatibility in several *in vitro* studies involving primary skeletal muscle cells.^{13,14} Although the scaffold mechanical properties are too large for native skeletal muscle currently, it is necessary to determine whether the materials in these composite scaffolds are *in vivo* biocompatible before further research into optimizing the scaffold is completed.^{13,14} Two variations of the PCL-MWCNT-(83/17, 40/60) PAA/PVA scaffold will be implanted within the skeletal muscle of rats to test for *in vivo* biocompatibility. We hypothesize that there will be a difference in biocompatibility with the two types of scaffolds tested.

Materials and Methods

Scaffold fabrication and preparation

All solutions were made as described previously to create 25% PCL-0.05% MWCNT-6% (83/17, 40/60) PAA/PVA scaffolds.^{13–15} The coaxial electrospinning parameters were a 5.00 mL/h extrusion rate, a 15 cm distance between the needle and mandrel, and a negative voltage of -2 kV. The positive voltage ranged from 11 to 15 kV for the 83/17 solutions and from 12 to 17 kV for the 40/60 solutions. All scaffolds were collected on a 5-cm-diameter mandrel rotating at $\sim 3000 \pm 200$ rpm. The PVA was crosslinked by soaking in a glutaraldehyde solution.²⁴ Scaffold segments for the 83/17 and 40/60 groups were cut into 5×3 mm pieces. These were sterilized by soaking in ethanol, exposure to UV

light on each side, and stored in sterile phosphate-buffered saline (PBS) as previously described.^{13,14}

In vivo scaffold implantation

All study procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee and were performed in a GLP compliant laboratory. For this portion of the study, 36 male, Sprague-Dawley rats weighing 175–200 g were used. Rats were housed in pairs in an alternating 12-h light/12-h dark cycle and fed commercial rat chow ad libitum. Rats were randomly numbered and assigned to an implantation group before being premedicated with 0.03 mg/kg of buprenorphine administered subcutaneously (SC), and then anesthetized with isoflurane in an induction chamber. Anesthesia was maintained through delivery of isoflurane and oxygen through a nosecone. A random number generator (EasyFit for Excel; Microsoft, Redmond, WA) was used to assign the two investigators (J.H.R. or K.M.F.) performing the scaffold implantations an equal number of surgical procedures per group.

The hair overlying the lateral aspect of the thigh was clipped and aseptically prepared using betadine and alcohol. A 2-cm-long skin incision was created in the craniolateral thigh area, exposing the biceps femoris muscle. The fascia and fibers of the biceps femoris were bluntly divided to expose the underlying vastus lateralis (VL) muscle. The fascicles of the VL were bluntly divided in a direction parallel to their long axis orientation to create an ~ 5 mm wide by 5 mm deep cavity within the muscle belly into which the scaffold segment was placed so that the scaffold fibers and VL myofibers were oriented in parallel. The surgical wound was closed routinely with a 4-0 polydioxanone (PDS) suture in a simple interrupted, intradermal pattern. For operated control limbs, the same procedure was repeated except that 0.1 mL of sterile PBS was injected into the muscle cavity in lieu of scaffold implantation as a sham surgery. An unoperated VL muscle was used as a control (Table 1). Although five rats were assigned to each group, only four were treated initially and the fifth rat was held in reserve in case complications arose.

Postoperative monitoring

Each rat received 0.03 mg/kg of buprenorphine administered SC every 12 h for 1 day after surgery. For the first week

TABLE 1. EXPERIMENTAL TREATMENT MATRIX

Rat group	Rat number	Hindlimb treatment		Time of sacrifice (Days postsurgery)
		Right	Left	
1	5	25% PCL-0.05% MWCNT-6% (83/17) PAA/PVA Scaffold	PBS Sham	7
	5	25% PCL-0.05% MWCNT-6% (83/17) PAA/PVA Scaffold	PBS Sham	14
	5	25% PCL-0.05% MWCNT-6% (83/17) PAA/PVA Scaffold	PBS Sham	21
	5	25% PCL-0.05% MWCNT-6% (83/17) PAA/PVA Scaffold	PBS Sham	28
2	5	25% PCL-0.05% MWCNT-6% (40/60) PAA/PVA Scaffold	Unoperated control	7
	5	25% PCL-0.05% MWCNT-6% (40/60) PAA/PVA Scaffold	Unoperated control	14
	5	25% PCL-0.05% MWCNT-6% (40/60) PAA/PVA Scaffold	Unoperated control	21
	5	25% PCL-0.05% MWCNT-6% (40/60) PAA/PVA Scaffold	Unoperated control	28
Total rats	40			

PCL, poly(ϵ -caprolactone); MWCNT, multiwalled carbon nanotubes; PAA/PVA, polyacrylic acid/polyvinyl alcohol; PBS, phosphate buffered saline.

following surgery, rats were clinically examined twice daily and their attitude, body weight, gait, and status of the surgical wound were recorded. For the rest of the 28-day period, the same clinical monitoring procedure was performed once daily.

Postmortem tissue collection and processing

On days 7, 14, 21, and 28 postimplantation, four rats from each treatment group were anesthetized with isoflurane as previously described and humanely sacrificed with an overdose of barbiturate anesthesia (phenytoin [50 mg/mL] and pentobarbital [390 mg/mL] at a dose of 1.5 mL/rat) administered intraperitoneally. Immediately following confirmation of death by cessation of spontaneous respiration and heartbeat accompanied by loss of the corneal reflex, 0.75 mL of blood was collected by cardiac puncture. Blood samples were harvested into serum separator tubes, allowed to clot for 30 min at room temperature, and centrifuged at 3000 g for 10 min. The serum was extracted, separated into 100- μ L aliquots, and frozen at -80°C . The treated and control quadriceps musculature from the hindlimbs of each rat was harvested in an en bloc fashion. The VL was severed at the insertion point followed by sharp dissection through the belly, 2 cm proximal to the area of scaffold implantation or control treatment, which was externally referenced to the proximal knot of the 4-0 PDS suture within the dermis.

Following en bloc removal of the VL muscle, the muscle was sharply divided through the center of the long axis of the implanted scaffold or through the center region of the PBS injection. A muscle section from every limb and treatment group was immersion fixed in 10% neutral buffered formalin. The second section was embedded in Optimal Cutting Temperature (OCT; Tissue Tek, Sakura Finetek USA, Torrance, CA) media, flash frozen in isopentane, precooled in liquid nitrogen, and stored at -80°C .

Quantitative and semiquantitative morphologic scaffold analyses

For all quantitative muscle pathologic observations, an observer blinded to the treatments obtained three high-power ($400\times$ magnification) digital photomicrographs from each treatment and staining procedure using a charge coupled digital camera (Nikon DS-Fi1, Nikon, Japan). Each photomicrograph was then numbered using a random number generator (EasyFit for Excel) so that investigators analyzing the data (K.M.F. and J.H.R.) were blinded to the treatments.

Postmortem macroscopic evaluation

Scaffold implantations were considered poor if they were traumatically explanted, migrated out of the implantation cavity, or $\leq 50\%$ of the surface area of the scaffold was in contact with myofibers within the implantation site. Implantations were graded as good if 50–90% of the scaffold surface area was in contact with myofibers surrounding the implantation cavity and the scaffold was adherent to the neighboring fascicles. Implantations were considered excellent if the scaffolds were firmly seated within the implantation cavity and $>90\%$ of the surface area of the scaffold was enveloped within the fascicular structure of the muscle upon harvesting.

Serum creatine kinase and lactate dehydrogenase enzyme analyses

Aliquots of serum were allowed to thaw at room temperature and activity assays for creatine kinase (CK) and total lactate dehydrogenase (LDH) were performed using a commercial automated biochemical analytical system (Olympus AU400; Olympus America, Inc., Melville, NY) according to the manufacturer's instructions in a GLP compliant commercial veterinary diagnostic laboratory ($n=4/\text{group}$). Four samples from rats that were not operated on were used as controls.

Morphologic evaluation of muscle tissue and scaffolds

Light microscopy. Muscle specimens fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (H&E), modified Masson's trichrome (TRI), or Verhoeff's Van Gieson (VVG) methods according to standard procedures.

Scaffold fibrosis. The surrounding fibrotic tissue was analyzed for the 83/17 scaffolds, 40/60 scaffolds, PBS-treated area, and the unoperated control muscle. ImageJ software (version 1.45s) was used to measure three values for the fibrotic tissue cross-sectional area for each sample from TRI-stained slides.²⁵ These values were then averaged together for final values for each treatment group per day.

Treatment-associated inflammation. Treatment-associated inflammation was semiquantitated following light microscopic visualization of the implantation area of H&E-stained slides using the following scoring system: (0) no treatment-associated inflammation visualized, (1) focal inflammation limited to the epimysium or perimysium surrounding implant cavity or scaffold, (2) multifocal or diffuse inflammatory infiltrates involving any of the connective tissue components surrounding the implant cavity, but sparing the myofibers or scaffold, (3) diffuse inflammatory infiltrates involving the epi-, peri-, endomysium, scaffold, and/or myofibers, and (4) diffuse, marked inflammatory infiltrates involving the epi-, peri-, endomysium, myofibers, and/or scaffold, associated with myofiber necrosis.

Scaffold cellularity. The total cell count on each scaffold was determined using two H&E-stained digital photographs for each implanted scaffold. Values for each scaffold type on each postimplantation day were averaged together. To determine the type of cell attaching to the scaffolds, a differential cell count was determined by enumerating 100 cells from digital photomicrographs of VVG-stained sections. Cells infiltrating the scaffold were counted and labeled based on the chromogenic appearance of their cytoplasm compared with control skeletal muscle as follows: elastic fibers (black), fibroblasts (pink/red), myogenic cells (yellow/tan), and unknown (indeterminant or unstained cells or nuclei).²⁶ Values for each cell type were then averaged together.

To further evaluate the possibility of myogenic scaffold cellular attachment and infiltration, frozen muscle samples from each rat were sectioned at 5 μm and immunohistochemically stained with primary antisera against desmin, a protein that is a myogenic marker, (rabbit polyclonal ab15200, 1:200 dilution; Abcam, Cambridge, MA)

according to the manufacturer's instructions.²⁷ The staining procedure was performed on an automated staining system (Benchmark XT; Ventana Medical Systems, Tucson, AZ) using an alkaline phosphatase (ALP) detection method and fast red counterstain. Commercial image analysis software (NIS-Elements AR, Nikon, Japan) was used to digitally threshold desmin immunoreactive cells from the background based on the mean desmin staining intensity of the positive control VL. Desmin immunoreactive cells clearly forming vascular profiles were excluded. The fraction of desmin immunoreactive cells/mm² over three fields was determined for each scaffold sample and averaged for a final value. These were then compared to the average number of VVG-stained myogenic cells/mm² in three fields as well.

Scaffold neovascularization. Muscle sections were sectioned at 5 μ m and immunohistochemically stained using primary antisera against CD31 (Clone TLD-3A12; 1:100 dilution, Millipore/Chemicon, Billerica, MA) and Factor VIII-related antigen: von Willebrand Factor (Factor VIII: vWF; rabbit polyclonal; 1:200 dilution; Millipore). All staining procedures occurred on an automated staining system (Benchmark XT; Ventana Medical Systems) using an ALP detection method and 3,3'-diaminobenzidine (CD31) or fast red counterstain (Factor VIII:vWF) according to the manufacturer's instructions. Commercial image analysis software (NIS-Elements AR) was used to digitally threshold Factor VIII:vWF immunoreactive cells from a background based on the mean Factor VIII:vWF staining intensity of the positive control VL. The number of Factor VIII:vWF immunoreactive, cross-sectional vascular profiles was counted from three high-power photomicrographs obtained from each scaffold sample.

Statistical analysis

Data were first checked for normality using the Shapiro-Wilks test and as the data were not normal, the Kruskal-Wallis nonparametric test was utilized to determine the

overall differences between groups. This analysis was performed using Stata software (StataCorp LP, College Station, TX). Then, Tukey's HSD one-way ANOVA method was used on the ranked data to determine the statistical significance using JMP software (SAS, Cary, NC). Data were considered statistically significant when $p < 0.05$.^{28,29}

Results

No intraoperative complications were observed during scaffold implantation, with all rats exhibiting normal behaviors and activities within 10 min of cessation of anesthesia. Minor postoperative complications were observed in six rats. Of these six, surgical wound dehiscence was observed in four rats within the first 12 h of surgery and remedied with topical application of tissue adhesive. It was believed that only one of these rats, one from the 40/60_d28 group, had lost the implanted scaffold and the fifth rat held in reserve was utilized. As it was believed that the other three rats had not lost their scaffold, no other surgeries were performed. Upon sacrifice, three of these four rats' dehiscence was associated with traumatic explantation of the scaffold, two rats from the 83/17_d14 group and one rat in the 83/17_d21 group. The rat from the 40/60_d28 group was found to not have lost the implanted scaffold and the final sample size for this group is $n = 5$. In addition, the scaffold was noted to have migrated out of the implantation cavity into the subcutaneous tissues of the thigh region in two rats (one 83/17_d7 and one 40/60_d14). The remaining two rats out of the six developed some mild, self-limiting incisional inflammation. No evidence of lameness or systemic illness was observed in any rat during the study.

Statistical analysis was initially completed on fibrotic tissue formation as two surgeons performed the scaffold implantations and it was found that there was no surgeon effect (data not shown). Therefore, for all subsequent analyses, only the treatment groups and sacrifice days will be compared. Scaffold implantations were graded on a 0–2 scale as scaffold loss/migration (0), good implantation (1),

FIG. 1. Representative digital micrographs displaying the scaffold implantation scoring system and fibrosis formation before explantation and after staining with hematoxylin and eosin (H&E). The bottom scale bar is 2 mm. Color images available online at www.liebertpub.com/tea

TABLE 2. AVERAGE IMPLANTATION SCORE VALUES WITH STANDARD DEVIATIONS FOR THE 83/17 AND 40/60 SCAFFOLDS

	83/17 Scaffolds	40/60 Scaffolds
Day 7	0.75 ± 0.50	1.25 ± 0.50
Day 14	0.75 ± 0.96	0.75 ± 0.50
Day 21	1.00 ± 0.82	1.00 ± 0.00
Day 28	1.25 ± 0.50	1.80 ± 0.45

or excellent implantation (2) (Fig. 1). Table 2 displays the average implantation score for the 83/17 and 40/60 scaffolds on the four sacrifice days. The calculated values were either one or close to a value of one and no significant differences were found.

Average CK and LDH values for the scaffold-implanted rats and rats that were not operated on as a baseline control are reported. The baseline control level for CK was 199 ± 9 and 279 ± 11 U/L for LDH. CK values on day 7 for the 83/17 and 40/60 implanted rats were 292 ± 21 and 339 ± 31 U/L, respectively. LDH values on day 7 were 374 ± 26 U/L for 83/17 implanted rats and 452 ± 39 U/L for 40/60 implanted ones. Both the CK and LDH values were significantly elevated on day 7 for both scaffold-implanted groups compared with the three other time points and the control. By day 14, the CK and LDH levels had decreased and were not significantly different from the baseline control average. The remaining two time points were also not significantly different from the baseline control for both scaffold-implanted groups.

During scaffold explantation, no degradation of the scaffolds was discerned. A subjective gross examination of the implants suggested that the 40/60 scaffolds induced larger amounts of fibrotic tissue. Figure 2 shows H&E- and TRI-stained images for the 83/17 scaffolds, 40/60 scaffolds, and PBS injected VL muscle on days 7 and 28. For comparison purposes, muscle harvested from rats that were not operated on and unoperated control VL muscle are also

shown (Fig. 2). Both scaffolds induced a broad inflammatory response accompanied by collagen deposition around the scaffold. However, the amount of fibrotic collagen tissue deposition seemed to decrease over time. When the average fibrotic tissue cross-sectional area was measured, only the 40/60_d7 was significantly greater than all other values (Fig. 3A). Both the 83/17 and 40/60 values decrease after day 7 and then plateau for the rest of the study. The PBS sham surgery values remain fairly consistent over the course of the study and as expected, the unoperated control muscle has the lowest values of all the groups.

Each of the four treatment groups was then graded on a scale from 0 to 3 to determine the amount of inflammation (Fig. 3B). The 40/60_d7 average values were significantly larger than all others in the study. Values for 83/17_d7 were larger than all other 83/17 time points and 40/60_d14 were larger than days 21 and 28. Cellular inflammatory infiltrates were predominantly composed of polymorphonuclear leukocytes with lesser numbers of lymphocytes in both types of scaffolds. As expected, the unoperated control had no inflammation and was significantly lower than the other three treatment groups except for PBS_d21, PBS_d28, and 83/17_d28.

Figure 4 demonstrates cellular infiltration of the 83/17 and 40/60 scaffolds during the 4-week study. Table 3 shows an increase in total cell number over the study period. The 83/17_d28 cell counts are significantly larger than 83/17_d7 ones. In addition, the 40/60_d28 is significantly greater than all other 40/60 values. The average number of myogenic cells, fibroblasts, cells producing elastin (most likely fibroblasts),³⁰ and unknown/indeterminant ones was assessed using the VVG stain (Fig. 5). The number of myogenic cells did increase over time and 83/17_d28 had the largest amount compared with all other values. Three values, 83/17_d21, 40/60_d21, and 40/60_d28, had a larger number of myogenic cells compared with days 7 and 14. In conjunction with the temporal increase in myogenic cell number, the number of fibroblasts per scaffold decreases over time. The 83/17_d28 scaffolds were significantly lower than all other

FIG. 2. Images of the 83/17 scaffolds, 40/60 scaffolds, and PBS sham surgeries on days 7 and 28 stained with H&E and trichrome (TRI) are shown. Controls of unimplanted scaffolds and unoperated vastus lateralis (VL) muscles are also shown. Scale bars represent 200 μ m in all panels.

values. Scaffolds for 83/17_d14 were lower than day 7 ones and 40/60_d14. A significant decrease was also seen on day 21 as both scaffolds were significantly lower than days 7 and 14. There were no significant differences for the elastin producing cells counted. However, the unknown cells on day 28 are significantly higher than both day 7 values and 40/60_d14. The 83/17_d14 unknown cell number is also significantly larger than both day 7 values and 40/60_d14.

Desmin positively stained myocytes on both types of scaffolds during the 4-week study are shown in Figure 6. Table 4 displays the average number of desmin immunoreactive cells/ mm^2 on each scaffold compared with the average number of VVG-stained myocytes/ mm^2 . Once again, the 83/17_d28 was larger than all other values. Values for desmin 83/17_d21 scaffolds, 40/60_d21 scaffolds, 40/60_d28 scaffolds, and VVG 83/17_d28 scaffolds were significantly greater compared with day 7 values, day 14 val-

ues, VVG day 21 values, and VVG 40/60_d28. VVG 40/60_d28, 40/60_d21, and 83/17_d21 were significantly larger than days 7 and 14.

Both the 83/17 and 40/60 scaffolds displayed evidence of neovascularization as early as day 7 after implantation. Figure 7A shows marked neovascularization of both scaffold types on day 28 as demonstrated with TRI-, CD31-, and Factor VIII:vWF-stained images. Figure 7B displays the average number of blood vessels for the 83/17 and 40/60 scaffolds on each sacrifice day. Although both the 83/17 and 40/20 scaffolds show an increase in the number of blood vessels over time, the 40/60 scaffolds have a larger increasing trend. The 40/60_d21 scaffolds are significantly greater than 83/17 scaffolds on day 7, 14, and 21. On day 28, the 40/60 scaffolds are significantly higher than all the other values. Although the 83/17 scaffolds had less vascularization on day 28 compared with 40/60, they are significantly larger than all other 83/17 values.

FIG. 4. 83/17 and 40/60 scaffolds stained with H&E to show the progression of cellular infiltration over the 4-week study period. Both scaffold types were also Verhoeff's Van Gieson (VVG) stained to differentiate between myogenic cells (yellow) and fibroblasts (pink). A control of unoperated VL muscle showing areas with small and large amounts of connective tissue between fascicles is also provided. All scale bars represent 200 μm .

TABLE 3. THE AVERAGE TOTAL NUMBER OF CELLS PER SCAFFOLD WITH STANDARD DEVIATIONS FOR THE FOUR TIME POINTS

	83/17 Scaffolds	40/60 Scaffolds
Day 7	20.00 ± 4.34	13.38 ± 8.42
Day 14	40.00 ± 11.60	10.33 ± 8.33
Day 21	54.67 ± 21.47	27.38 ± 21.94
Day 28	78.88 ± 35.19 ^a	73.80 ± 56.58 ^b

^{a,b} $p < 0.05$.

Discussion

In this study, PCL-MWCNT-(83/17, 40/60) PAA/PVA scaffolds were successfully implanted into a cavity created within the VL muscle of rats. Scaffold implantation was not associated with any significant clinical adverse effects and complications associated with the implantation procedure were minimal. After the surgical procedure, rats were ambulatory within 10 min of anesthesia and normal activity was recorded for the 4-week time period. Figure 1 displays representative images of the scaffold grades, while Table 2 gives the average scaffold score. It was determined that the scaffolds were scored as a good implantation or were close to this value as there were no significant differences. This means that the procedures were performed well and one less variable exists when comparing between samples. However, some scaffold loss or migration out of the implant cavity did occur. Although numerous methods for securing the scaffolds may have prevented this, the purpose of the study was to determine scaffold biocompatibility. Thus, we wished to avoid complicating variables such as suture or tissue adhesive reactions or coimplantation of biologically inert an-

choring devices. Future studies should consider methods to securing the scaffold in place.

CK and LDH are enzymatic markers of tissue damage. Levels for CK and LDH were significantly elevated on day 7 compared with unoperated control values indicating that there was some skeletal muscle damage. By day 14, both enzyme levels had returned to baseline values in both scaffold groups. In comparison, Makinen *et al.* measured normal CK and LDH values for male Sprague-Dawley rats as 190.7 ± 45.5 and 167.9 ± 20.0 U/L, respectively.³¹ Our untreated CK value, 199.25 ± 8.96 U/L, overlaps the range given, but the LDH value, 279.25 ± 10.78 U/L, does not. The observation of the day 7 myocellular enzymatic increase is not unexpected considering the finding of morphologic evidence of maximum inflammation and fibrosis at day 7.

Although no degradation of the scaffolds was expected or observed, future work needs to include analyzing the degradation of the PAA/PVA hydrogel. After measuring the cross-sectional area of the fibrotic capsule, only the 40/60_d7 was significantly greater. After day 7, the cross-sectional area measured for both scaffolds decreases and then remains fairly constant. The response from the PBS sham surgeries shows slightly elevated levels of fibrotic tissue compared with the unoperated control. The PBS sham surgeries have less capsule formation compared with the 40/60 scaffolds, but the 83/17 scaffolds have a similar amount after day 7. As the amount of fibrotic capsule decreases after day 7, we conclude that our PCL-MWCNT-PAA/PVA scaffolds are not considered hazardous by the body. Although the fibrotic tissue amount decreased during the study, the scaffold could be soaked in growth factors to further reduce fibrosis and promote muscle regeneration. Sato *et al.* delivered insulin-like growth factor-1 and decorin, an anti-TGF- β human proteoglycan, after muscle injury. Although

FIG. 5. The number of (A) myogenic cells, (B) fibroblasts, (C) elastin producing cells, and (D) indeterminate cells per 100 for the 83/17 and 40/60 scaffold sections with standard deviations stained using VVG (*, #, % $p < 0.05$).

FIG. 6. Digital photomicrographs of the 83/17 and 40/60 scaffolds stained for desmin-positive cells (dark pink/red) over the 4-week period. Rat colon serves as the positive rat control.

both molecules decreased fibrotic tissue formation compared with the sham control, the decorin-treated muscle had significantly lower amounts.³²

The largest amount of inflammation was observed on day 7 by both scaffolds with the 40/60_d7 values being significantly larger than all others. Overall, the 40/60 scaffolds

elicited a larger response than the 83/17 scaffolds. The 83/17 scaffold_d28 values were not significantly different from the PBS_d21, PBS_d28, and all unoperated VL controls. Although both scaffolds induced an inflammatory response, it does decrease during the 4-week period. Boennelycke *et al.* cultured muscle-derived cells or muscle fiber fragments on methoxypolyethyleneglycol-poly(lactic-co-glycolic acid) (MPEG-PLGA) scaffolds and implanted them superficially to the abdominal muscle fascia.¹⁰ At 3 weeks postimplantation, inflammatory scores were qualitatively assessed as 3 or moderate and by 8 weeks had decreased to 1 or minimal.^{10,33} However, our inflammatory response was more similar to the acellular skeletal muscle scaffolds implanted into the skeletal muscle of mice reported by Perniconi *et al.* They found that there was an overt inflammatory response 2 weeks postimplantation, but by 4 weeks it had decreased toward control levels.⁹ Longer studies are necessary to determine whether the inflammation caused by the implantation of the scaffold continues to decrease over time.

The total number of cells attached to the 83/17 and 40/60 scaffolds were counted and the values displayed in Table 3. Cells infiltrated the scaffolds and the number of cells increased over time with the most cells attached on day 28. As there was fibrotic tissue surrounding the scaffold, the type of cell attached to the scaffold was in question. Sections were stained with VVG to determine whether the cells were myogenic, fibroblasts, or elastin producing. It was found that the number of fibroblasts attached decreased and the number of myogenic cells increased during the 4-week period (Fig. 5). However, some cell types were unable to be determined with the VVG stain, so a desmin immunohistochemistry was used to more effectively determine the number of myogenic ones. Table 4 shows that both the desmin stain and the VVG stain results show an increase in the number of myocytes over time. However, the desmin-stained cells displayed a larger number at each of the time points except when the desmin 40/60_d28 value was compared with the VVG 83/17_d28 value. Our initial results show that some myogenic-like cells, skeletal muscle cells, or myofibroblasts, are attaching and infiltrating the scaffolds; however, future work will focus on using a more detailed analysis to determine what type of myogenic cell is present. Although no myofibers could be conclusively determined, future work aims for longer implantation periods to ascertain whether the myogenic cells will organize into myofibers.

FIG. 7. (A) TRI, CD31, and FactorVIII: von Willebrand factor-stained images for 83/17 scaffolds, 40/60 scaffolds, and unoperated control VL muscle. Black arrows highlight some of the scaffold neovascularization on the TRI-stained images. Scale bars represent 200 μm in all images. (B) The average number of blood vessels with standard deviations for the 83/17 and 40/60 scaffolds over the 4-week period *, #, % $p < 0.05$.

TABLE 4. AVERAGE DESMIN ANTIBODY- AND VVG-STAINED MYOGENIC CELLS/MM² WITH STANDARD DEVIATIONS ON THE 83/17 AND 40/60 SCAFFOLDS

	Desmin Ab stain		VVG stain	
	83/17 Scaffolds	40/60 Scaffolds	83/17 Scaffolds	40/60 Scaffolds
Day 7	13.67 ± 4.66	14.00 ± 5.05	1.11 ± 1.05	1.00 ± 1.04
Day 14	46.17 ± 5.81	51.75 ± 6.84	40.50 ± 3.15	38.50 ± 11.67
Day 21	181.00 ± 15.45 ^a	190.08 ± 16.19 ^a	117.22 ± 14.73 ^b	120.92 ± 22.75 ^b
Day 28	328.25 ± 19.37 ^c	188.07 ± 14.39 ^a	189.58 ± 12.35 ^a	115.27 ± 15.66 ^b

^{a-c}*p* < 0.05.

VVG, Verhoeff's Van Gieson.

Digital photomicrographs of TRI-stained 83/17 and 40/60 scaffolds showed evidence of neovascularization (Fig. 7). Both scaffolds show an increase in the number of blood vessels during the study; however, the 40/60 scaffolds have a higher trend and are significantly greater than all other values on day 28. Values for the 83/17_d28 scaffolds are also significantly greater than the other 83/17 values (Fig. 7). Levenberg *et al.* investigated vascularization of skeletal muscle by culturing myoblasts, embryonic fibroblasts, and endothelial cells on poly(L-lactic acid) (PLLA)-PLGA scaffolds *in vitro*. After 2 weeks of culture, the control that was untreated with growth factors had an approximate average value of three blood vessels/mm².³ Our 2-week average value for 40/60 scaffolds, 1.78 ± 1.09 blood vessels/scaffold, is similar, but the 83/17_d14 value is not as it was zero. They also found that the embryonic fibroblasts differentiated into smooth muscle cells and surrounded the endothelial cells to form vessel-like structures.³ More investigation is necessary to determine whether the decrease in fibroblasts attached to the scaffolds coupled with the increase in blood vessels is related to fibroblast differentiation into smooth muscle cells. Although vascularization occurs, the vessels were not completely aligned perpendicular to the scaffold fibers as naturally occurring vessels are to skeletal muscle. Future studies could have small areas without hydrogel in the scaffold to provide a path of least resistance for the vessels to obtain better perpendicular alignment. In addition, growth factors encapsulated within microspheres such as the vascular endothelial growth factor could be electrospun with the PCL-MWCNT solution to aid in guiding the vessel through the scaffold.³

Conclusion

PCL-MWCNT-(83/17, 40/60) PAA/PVA actuating scaffolds that tested well under *in vitro* conditions were implanted in rat skeletal muscle for 4 weeks to determine *in vivo* biocompatibility. These two types of scaffolds displayed early signs of fibrotic tissue formation, inflammation, and elevated levels of CK and LDH, but these responses decreased during the study. Cells were found to have infiltrated both scaffolds and the cell type was determined through histological staining. Early time points showed more fibroblasts attached to the scaffolds, but over time, the number of fibroblasts decreased, while the number of myogenic cells increased. Both scaffolds also displayed evidence of neovascularization. We conclude that both our unique PCL-MWCNT-PAA/PVA scaffolds were biocom-

patible; however, some differences between the two scaffold types were observed. The 83/17 scaffolds had more myogenic cells attached to the scaffold on day 28, fewer fibroblasts attached, a smaller fibrotic capsule, and less inflammation. In comparison, the 40/60 scaffolds had fewer attached myogenic cells, more fibroblasts attached, a larger fibrotic capsule, and a larger inflammatory response. However, over time, the myogenic cell number increased, while the number of attached fibroblasts decreased. The inflammatory response and fibrotic capsule also decreased during the study. For scaffold neovascularization, the 40/60 scaffolds had a greater amount compared with the 83/17 scaffolds. Although initial results are encouraging, longer studies are necessary to determine if the two hydrogel concentrations will critically affect the body, including local and systemic effects. Future studies will investigate the scaffold's ability to regenerate skeletal muscle after injury.

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Disclosure Statement

No competing financial interests exist.

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