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Engineered Porcine-derived Urinary Bladder Matrix Conduits as a Novel Scaffold for Peripheral Nerve Regeneration

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Abstract

Purpose—This study aims to compare engineered nerve conduits constructed from porcine-derived urinary bladder matrix (UBM) with the criterion-standard nerve autografts, for segmental loss peripheral nerve repairs.

Methods—Forty-eight Sprague-Dawley rats were divided into 2 groups. All underwent a 10-mm sciatic nerve gap injury. This was repaired using either (1) reverse autograft—the 10-mm cut segment was oriented 180 degrees and used to coapt the proximal and distal stumps or (2) UBM conduit—the 10-mm nerve gap was bridged with UBM conduit. Behavior assessments such as sciatic function index and foot fault asymmetry scores were performed weekly. At 3- or 6-week time endpoints, the repaired nerves and bilateral gastrocnemius/soleus muscles were harvested from each animal. Nerves were evaluated using immunohistochemistry for motor and sensory axon staining and with diffusion tensor imaging. The net wet muscle weights were calculated to assess the degree of muscle atrophy.

Results—The UBM group demonstrated significantly improved foot fault asymmetry scores at 2 and 4 weeks, whereas there was no difference in sciatic function index. The net muscle weights were similar between both groups. Motor axon counts proximal/inside/distal to the conduit/graft were similar between UBM conduits and reverse autografts, whereas sensory axon counts within and distal to the conduit were significantly higher than those of the autograft at 6 weeks. Sensory axonal regeneration seemed to be adherent to the inner surface of the UBM conduit, whereas it had a scattered appearance in autografts. Diffusion tensor imaging parameters between groups were similar.

Conclusions—Urinary bladder matrix conduits prove to be at least similar to nerve autografts for the repair of peripheral nerve injuries with a short gap. The matrix perhaps serves as a scaffold to augment sensory nerve growth.

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Clinical Relevance—In a clinical setting, UBM may eliminate the donor site morbidity and increased operative time associated with nerve autografting.

Keywords

conduit; diffusion tensor imaging; extracellular matrix; peripheral nerve; urinary bladder matrix

Most peripheral nerve injuries are traumatic in nature, and debridement of damaged nerves outside the zone of injury is often necessary for improved regeneration.¹ The potential nerve gap created results in difficulties to achieve tensionless primary neurorrhaphy.² Autografts are the criterion standard to treat segmental peripheral nerve gaps; however, autologous nerve grafting has been limited by donor site morbidity and the availability of expendable donor nerves. For this reason, various conduits have been studied as alternatives to autografts.^{3–5} Current Food and Drug Administration–approved biodegradable conduits in the market have had variable results in comparison with autografts both clinically and experimentally. These variable results have precluded conduit-based repair full acceptance in bridging segmental peripheral nerve loss,^{2,3,6,7} leading bioengineers continue to search for conduit material that will consistently outperform autografts.

Extracellular matrix (ECM) has been studied as a tissue scaffold and has demonstrated favorable outcomes in many reconstructive fields.^{8–12} It has a complex array of functional proteins, such as collagen and laminin, that provides structural support and serves as an orchestrator to signaling molecules for cell growth and differentiation, angiogenesis, and modulates immune cells for wound healing.^{8,13} Because of its versatility and benefits seen in wound healing, we study conduits constructed of ECM derived from porcine urinary bladder for the repair of peripheral nerves with gaps.

METHODS

Experimental Design/Animal Model

Adult female Sprague-Dawley rats (n = 48; 200–250 g; Charles River Lab) were used in the experiment. The objective of this study is to evaluate behavior, histology, and diffusion tensor imaging (DTI) parameters between 2 groups: (1) autologous grafts (n = 24) and (2) conduits constructed of porcine-derived urinary bladder matrix (UBM) (n = 24) for the repair of peripheral nerve gaps in a rat sciatic nerve model. All rats underwent postoperative behavioral studies. After reaching 3- or 6-week time endpoints, the left sciatic nerve was harvested and evaluated with (1) immunohistochemistry (IHC) or (2) DTI. Additionally, bilateral gastrocnemius/soleus muscles were harvested to obtain net wet weights (Fig. 1).

Rats were housed in a central animal care facility and were provided food and water ad libitum. All experimental procedures were approved and performed in accordance with the standards of our institutional animal care and use committee.

Sciatic Nerve Injury

Each rat was anesthetized via inhaled isoflurane (2%). The left hindquarter prepped aseptically. A 3-cm incision was made at the left sciatic notch, parallel and posterior to the

left femur. The biceps femoris was then reflected to expose the sciatic nerve. A 10-mm nerve gap was created in the midportion of the sciatic nerve with microsurgical scissors. The gap was repaired with either of the following: (1) The transected portion of the nerve was reversed 180 degrees and used as an autograft to coapt the proximal and distal stumps using 9–0 nylon suture (Ethicon, Somerville, NJ) in an end-to-end fashion using microsurgical technique. (2) A 10 mm (L) × 1.5 mm (D) UBM conduit was used to bridge the sciatic nerve gap and secured using 9–0 nylon sutures. The muscle and skin were closed to cover the nerve using 5–0 monocryl suture (Ethicon).

Behavioral Testing

Postoperatively, foot fault (FF) asymmetry and sciatic functional index (SFI) behavioral assessments were performed at weekly intervals. To prevent possible bias, behavior testers were blinded to the surgical protocols of each animal.

Foot Fault

Animals were allowed to roam freely on a wire mesh grid measuring 45 × 30 cm, with square openings measuring 2.5 × 2.5 cm. The grid was elevated 2 cm above a solid base. Trials of 50 total steps per hind limb were recorded for each animal. A full fault was recorded if the animal's hind limb fell through the opening in the grid and touched the floor, whereas a partial fault was recorded if the foot did not touch the floor. An FF asymmetry score was calculated using the following equation: composite FF score = (n partial faults × 1) + (n full faults × 2), %FF = (composite FF score/total number of steps) × 100%, FF asymmetry score = %FF (normal hind limb) – %FF (surgical hind limb).

Sciatic Functional Index

Before surgery, habituation trials were performed for animals to navigate an inclined beam into their home cage without hesitation.¹⁴ Each hind limb was inked, and animals walked the beam until 6 consecutive (3 from each limb) foot prints were recorded. Print measurements include the following: normal print length (NPL), normal toe spread (NTS), normal intermediary toe spread (NIT), experimental print length (EPL), experimental toe spread (ETS), and experimental intermediary toe spread (EIT). Sciatic functional index scores were the calculated using the following formula: $SFI = -38.3([EPL - NPL]/NPL) + 109.5 ([ETS - NTS]/NTS) + 13.3([EIT - NIT]/NIT) - 8.8$.^{15,16}

Sciatic Nerve and Gastrocnemius/Soleus Muscle Harvest

When the animals reached their final time endpoint, they were euthanized with Euthasol (Virbac AH, Fort Worth, Tex) intracardiac injections. The full length of the nerve was harvested and distributed equally to undergo either DTI processing or histology via IHC staining. The gastrocnemius and soleus muscles were then dissected from each hind limb and weighed (in grams) to obtain net wet weights for each animal.

Histology

Immunohistochemical staining for motor and sensory axons was performed as previously described using commercial antibodies specifically directed against carbonic anhydrase II

(Abcam, Cambridge, Mass) and choline acetyltransferase (Millipore, Temecula, Calif).¹⁷ For carbonic anhydrase II, primary antibody was used at 1:200 overnight. For choline acetyltransferase staining, primary antibody was used at 1:200 for 1 hour. Digital images of stained sections were acquired using Image-Pro Plus 7.0 software (Media Cybernetics, Bethesda, Md) with an Olympus C-35AD-4 microscope at low magnification (10×). Digital images were loaded on ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda, Md); however, axon counts were obtained by manual count of the entire nerve area.

Diffusion Tensor Imaging

Tissue Sample Preparation—After nerve excision, reverse autografts were incubated in 4% glutaraldehyde/0.5% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C and nerves repaired with a UBM conduit were incubated in 4% glutaraldehyde/2% paraformaldehyde in PBS at 4°C for better penetration of the conduit material. After a minimum of 24 hours of postfixation, excised nerves were placed in PBS + 2 mM gadolinium diethylene triamine pentaacetic acid (Magnevist, Bayer HealthCare, Wayne, NJ) at 4°C for at least 24 hours before imaging. For imaging, excised nerves were placed in glass capillary tubes (3-mm outer diameter) filled with a perfluoropolyether liquid (Fomblin, Solvay Solexis, Thorofare, NJ) for susceptibility matching, preventing tissue dehydration, and a signal-free background. For higher throughput, 6 nerves in a hexagonal arrangement were imaged simultaneously.

Diffusion Tensor Magnetic Resonance Imaging—Magnetic resonance imaging was performed on a 4.7-T Agilent Direct Drive scanner (Agilent Technologies, Santa Clara, Calif) for reverse autografts and a 9.4-T Agilent Direct Drive scanner for conduit-repaired nerves. Diffusion tensor imaging data were acquired using a 3-dimensional diffusion-weighted spin-echo sequence with repetition time/echo time of 170/23.0 ms, 12 signal averages, and field of view of $9.6 \times 9.6 \times 14.4 \text{ mm}^3$ for reverse autografts and repetition time/echo time of 160/23.0 ms, 10 signal averages, and field of view of $9.6 \times 9.6 \times 18.0 \text{ mm}^3$ for conduit-repaired nerves. The nominal resolution for both groups was $100 \times 100 \times 450 \text{ }\mu\text{m}^3$ (450 μm along the nerve). Diffusion weighting was achieved with $\delta/\tau = 4/12 \text{ ms}$, a prescribed b value of 2000 s/mm^2 , and 6 directions. One b = 0 image was acquired for a total of 7 images in a scan time of approximately 12 hours.

DTI Post-processing and Analysis—Image data reconstruction was performed using in-house written code in MATLAB (MathWorks, Natick, Mass), and DTI analysis was implemented using ExploreDTI.¹⁸ Three-dimensional image volumes were zero-padded 2× in each direction during reconstruction from k-space data. Diffusion tensors were estimated voxel-wise using a linear least-squares approach. From the diffusion tensor, fractional anisotropy (FA), axial diffusivity (AD), and radial diffusivity (RD) were computed on a voxel-wise basis and DTI tractography was performed.

Statistical Analysis

Statistical significance was determined using nonparametric Mann-Whitney U test and designated a *P* value of less than 0.05.

RESULTS

Behavioral Evaluations of Sciatic Nerve Function

Animals repaired with a UBM conduit significantly performed better in FF than the reverse autografts in the 2-week and 4-week time point (Fig. 2). When evaluating SFI scoring, the UBM conduit had behavior results comparable with the reverse autograft at all time points (Fig. 3).

Gastrocnemius/Soleus Net Wet Weights

There was no statistically significant difference in net muscle weight between the UBM conduit and autografts at 3 weeks or 6 weeks (Fig. 4).

Nerve Histology With IHC

When harvesting the injured nerves, the UBM conduit segment in the 6-week endpoint group seemed more similar in size to the proximal and distal ends of the sciatic nerve than the 3-week conduits. The 3-week conduits seemed bulkier. This was accounted for by partial biodegradation of the conduit in 6-week group compared with the 3-week animals. No samples demonstrated evidence of neuroma formation.

After staining with choline acetyltransferase for motor axons and carbonic anhydrase II for sensory axons, cross sections proximal to, within, and distal to the conduit/graft were evaluated. There was no significant difference in motor axon counts proximal to, within, and distal to the conduit/graft in UBM conduits and autografts in the 6-week time point (Fig. 5a). However, the UBM conduit had greater sensory axons within the conduit (455 ± 31 vs 140 ± 34 , $P < 0.01$) and distal to the conduit (253 ± 27 vs 77 ± 14 , $P < 0.01$) in comparison with the autograft group at 6 weeks (Fig. 5b).

Within the 3-week time point, UBM conduits had similar axon counts to the reverse autografts in both motor and sensory axons proximal and within the conduit/graft. However, it is important to note that 2 samples in the 3-week UBM conduit group had uncharacteristically lower sensory counts compared with the other 3-week UBM samples (but similar to autografts) because of collapse, which resulted in a loss of statistical significance. Because of uncleared cellular debris from Wallerian degeneration in the distal end at 3 weeks, axon counts at those segments for both study groups were deemed unreliable and, thus, were not included in this study.

Motor axons in both groups seemed to be uniformly distributed in all segments (Fig. 6a). Interestingly, the sensory axons within the UBM conduit seemed to grow adherent to the inner lining of the conduit and then demonstrated a scattered appearance once they reached the distal segment. Sensory axons in the reverse autografts seemed to be scattered in both the graft and distal segments (Fig. 6b).

Diffusion Tensor Imaging

Diffusion tensor imaging of excised sciatic nerves was evaluated in 3 regions of interest: 1.5 mm proximal to the proximal graft site, the middle of the conduit/autograft, and 1.5 mm

distal to the distal graft site. Fractional anisotropy, AD, and RD were measured for each region of interest. Within the 3- or 6-week UBM group, there was no difference in FA, AD, and RD, in all segments compared with the autografts (Table 1). Some UBM conduit samples had evidence of partial or near complete collapse without visualization of distal tracks. Figure 7 shows tractography demonstrating proximo-distal axonal growth at 6 weeks for noncollapsed UBM conduits and reverse autografts.

DISCUSSION

With advances in understanding nerve regeneration pathophysiology, tissue engineering research has focused on developing anisotropic ECM scaffolds that augment axonal regeneration and Schwann cell migration.¹⁹ In this study, we evaluate ECM derived from porcine bladder matrix to serve as a novel scaffold. In comparison with other xenogeneic ECM, porcine UBM has demonstrated fewer invasions of fibroblasts because of its intact basement membrane complex on its surface, thereby decreasing scar tissue formation.⁸ The advantage of an intact basement membrane complex also helps regulate cell growth, differentiation, and migration during tissue development and reconstruction.^{7,8}

The beneficial regenerative aspects of UBM scaffolds were demonstrated in this study because we found improved sensory axon numbers in comparison with the autograft. Uniquely, sensory axons also seemed adherent to the inner surface lining of the UBM conduit. Although the mechanism in which sensory axons favor growth along the conduit is unknown, structural proteins (ie, laminin, fibronectin, collagen) known to be present in ECM may be responsible. In vitro assays on human fetal sensory neurons have shown that laminin enhances Schwann cell response and antibodies to laminin suppress sensory neurite growth.^{20,21} Similarly, fibronectin has been shown to act as a chemoattractant for migrating Schwann cells and provide a direct substrate for adhesion and outgrowth of regenerating sensory axons.^{20–23} The UBM conduit basement membrane may possess more of these ECM structural proteins that support Schwann cell and sensory axon infiltration over autografts; however, further studies would need to be performed to identify the causative factor. Additionally, motor axon growth was not significantly different between groups at both 3- and 6-week time points. In relation to motor axon regeneration, UBM conduits presumably have a similar structural milieu to autografts to augment axonal outgrowth.

Although histomorphological parameters give information regarding nerve regeneration on a microscopic level, it is important to translate this information to functional recovery as histological evaluation after repair is not possible in humans. The FF scoring system has historically been used to determine the sensorimotor function in animals.²⁴ The improved FF score at 2 and 4 weeks within the UBM conduit could be explained by higher sensory axon counts, in which animals may have more sensory feedback for the next step and grip the rung of the grid with fewer faults. However, despite a more robust sensory axon count, there is question of the accuracy of the FF at 2 weeks postoperatively, as axons are unlikely to have made complete functional connections.²⁵ De Medinaceli et al¹⁵ had described difficulty in evaluating sensory function because of overlapping innervation and indirect measurements. Therefore, SFI scoring is regarded as a more reliable and reproducible quantitative method for the assessment of functional outcome in the recovery of the sciatic

nerve in rats.^{14,15,26–28} In this study, we found that UBM conduits performed no worse than that of autografts in SFI at any time point.

The gastrocnemius net weight was similar between both groups, demonstrating likely similar degrees of muscle reinnervation. As expected, there was greater muscle atrophy in the injured limb in comparison with its control limb. Martins et al²⁹ described that one must consider that not all regenerated fibers are viable and misdirected axon growth can lead to aberrant muscle reinnervation. This consideration, in addition to the time it takes regenerating axons to reach the motor end plate, explains the difference in the degree of atrophy between the experimental and the control limb.

This study is the first to monitor the efficacy of a nerve conduit in nerve regeneration using DTI technology. DTI is an advanced neuro-imaging tool with the ability to evaluate peripheral nerves noninvasively, objectively, and quantitatively. Parameters yielded by DTI, such as FA, AD, and RD, reflect the anisotropic diffusion of water molecules along and perpendicular to the axon bundles, which provide valuable information on axonal regeneration and degeneration.³⁰ The UBM conduits performed equally in all parameters of DTI in comparison with autografts demonstrating similar axonal integrity in all time periods. Diffusion tensor imaging can further be expanded to create tractography images, which allow visualization of the longitudinal extent of nerve regeneration. This study demonstrated track formation proximally to distally in the UBM conduit similar to reverse autografts. However, because of partial collapse found in some UBM samples, further experiments will need to be performed with less deformable models.

The timing of outcome assessment in animal studies is critical to recognizing the true differences between study groups. Rodents have been described to have rapid neuroregenerative capacity compared with humans and higher mammals. Studying outcomes at earlier time points provide better clinical correlation as the experimental and control groups are still significantly different. Later time point (ie, 15 weeks) observations may show experimental and control groups to be equivalent because of the enhanced regenerative capacity of the rodent nervous system, and this could mask any differences seen between groups.^{3,6,31,32} At 6 weeks, Wallerian degeneration is complete, yet axonal regeneration is still ongoing, providing the ability to more accurately compare recovery differences between both groups. Therefore, this time point strengthens the validity of improved sensory axons at least early in the nerve recovery process when utilizing the UBM conduit. Further studies with later time points would need to be performed to ascertain if this still remains true when axonal regeneration is considered complete.

In comparing gross appearance of 3- and 6-week conduits groups, the latter conduits revealed that the conduit had partially degraded over time. A conduit resorption rate that mirrors the speed of nerve regeneration would be ideal as this would provide stability of the conduit walls for nerve regeneration, while being degradable to prevent scar tissue accumulation and nerve compression.^{7,33} These features were demonstrated in the UBM conduit as both axonal regeneration and conduit degradation occurred over time.

The need to develop an ideal conduit that can perform superiorly to autografts is important, particularly for the deficit seen in conduits for gaps over 30 mm.^{2,6,7} Furthermore, the diameter of the injured nerve and conduit employed in a repair significantly affects nerve regeneration because larger conduits may cause a dilution effect of neurotropic factors and are subject to collapse.^{2,3,6,25} Few studies have been conducted for large diameter nerve repairs, and many have reported the inferiority of the use of any conduit in comparison with autografts.^{3,6,7,34,35} In this study, we were able to demonstrate at least comparable results for segmental gaps of 10 mm with a conduit diameter of 1.5 mm. The need to study this model in larger gaps, with larger diameter nerves, is necessary to evaluate the UBM conduit's potential against autografts in larger animals.

In this study, UBM conduits demonstrated to be at least similar to nerve autografts for the repair of peripheral nerve injuries. The matrix perhaps serves as a scaffold to augment sensory nerve growth. In a clinical setting, these promising results may eliminate the donor site morbidity and increased operative time associated with nerve autografting.

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FIGURE 1.
Experimental design.

FIGURE 2. Foot fault scoring. * $P < 0.05$, statistically significant. A more negative score indicates worse impairment.

FIGURE 3. Sciatic function index scoring. A more negative score indicates worse impairment.

FIGURE 4.
Gastrocnemius/soleus muscle net wet weight.

FIGURE 5. A, Motor axon count at 6 weeks. B, Sensory axon count at 6 weeks. * $P < 0.05$, statistically significant.

FIGURE 6.

A, Motor axon histomorphology at 6 weeks. Motor axons seem to be uniformly distributed in all segments in both groups. B, Sensory axon histomorphology at 6 weeks. Sensory axons within the conduit seem to grow adherent to the inner lining of the UBM conduit.

FIGURE 7. Diffusion tensor imaging tractography at 6 weeks demonstrating nerve regeneration in (A) UBM conduits that did not collapse and (B) reverse autografts.

TABLE 1
Diffusion Tensor Imaging Parameters in Each Segment Between UBM Conduit and Reverse Autograft at 6 Weeks

	Proximal	SEM	P	Within	SEM	P	Distal	SEM	P
FA, $\mu\text{m}^2/\text{ms}$			0.57			1.00			0.08
UBM conduit	0.578	0.019		0.593	0.022		0.481	0.010	
Reverse autograft	0.524	0.016		0.562	0.014		0.545	0.018	
AD, $\mu\text{m}^2/\text{ms}$			0.57			0.08			0.57
UBM conduit	0.910	0.037		0.098	0.054		0.890	0.079	
Reverse autograft	0.082	0.026		0.800	0.038		0.791	0.020	
RD, $\mu\text{m}^2/\text{ms}$			1.00			0.08			0.57
UBM conduit	0.360	0.029		0.488	0.053		0.440	0.037	
Reverse autograft	0.387	0.021		0.357	0.028		0.363	0.015	

P < 0.05, statistical significance.