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Tissue Engineered Bone Differentiated From Human Adipose Derived Stem Cells Inhibit Posterolateral Fusion in an Athymic Rat Model

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Abstract

Study Design—Biological augmentation spinal arthrodesis trial in athymic rats

Objective—To assess the efficacy of tissue-engineered bone to promote L4–L5 intertransverse process fusion in an athymic rat model.

Summary of Background Data—Each year in the United States, over 400,000 spinal fusion surgeries are performed requiring bone graft. The current gold standard for posterolateral lumbar fusion is autogenous iliac crest bone graft (ICBG), but the harvesting of ICBG is associated with increased operative time and significant complications. This being the case, an alternative cost-effective bone graft source is needed.

Methods—Bovine bone cores were sterilized and decellularized for scaffold production. Human adipose derived mesenchymal stem cells (ADSC) were obtained and verified by tri-differentiation testing and seeded onto dried scaffolds. The seeded cores were cultured for five weeks in culture medium designed to mimic endochondral ossification and produce hypertrophic chondrocytes. Single-level intertransverse process fusions were performed at the L4–L5 level of 31 athymic rats. Fifteen rats were implanted with the hypertrophic chondrocyte seeded scaffold and sixteen had scaffold alone. Half of the study rats were sacrificed at 3 weeks and the other half at 6 weeks. Spinal fusion was assessed using 2D and 3D micro computed tomography (μCT) analysis as well as tissue histology.

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Level of Evidence: N/A

Results—At 3 weeks, none of the tissue engineered rats had partial or complete fusion, while 62.5% of the decellularized rats fused and another 12.5% had partial fusions ($p=0.013$). At 6 weeks, none of the tissue engineered rats fused and 50% had partial fusions, while 87.5% of the decellularized rats fused ($p=0.002$).

Conclusion—Tissue engineered bone composed of hypertrophic chondrocytes inhibits posterolateral fusion in an athymic rat model and therefore does not represent a promising cost-effective bone graft substitute.

Keywords

tissue engineered bone; stem cells; spinal fusion; spine surgery; chondrocytes; bone graft; bone substitute; athymic rat model

Introduction

Over 400,000 spinal fusion surgeries are performed each year in the United States to treat a variety of spinal conditions including deformity, spinal stenosis, spondylolisthesis, vertebral fracture, and degenerative disc disease.¹ The increasing prevalence of spinal fusion procedures may be attributable to osteoporosis and the aging population.^{1,2,3} In addition to decortication and stability, successful fusion is largely dependent on the utilization of bone graft and bone graft substitutes.^{4,5}

The current gold standard for posterolateral lumbar fusion is autogenous iliac crest bone graft (ICBG) due to its easy procurement as well as its osteogenic, osteoinductive, and osteoconductive properties.⁶ However, the use of ICBG is riddled with donor site morbidity including osteomyelitis, sensory disturbances, and chronic harvest-site pain.^{6,7,8} In fact, some patients experience a greater amount of pain from the harvest-site than from the spinal fusion procedure during the immediate post-operative period.⁹ Moreover, even with the use of autogenous ICBG, there is still a significant risk of pseudarthrosis. Studies have demonstrated a 8% risk of pseudarthrosis in nonsmokers and up to 40% in smokers undergoing short posterolateral intertransverse process fusions.¹⁰ Furthermore, autogenous ICBG is also limited by the quantity of bone that can be extracted, increased blood loss, and increased operative time¹¹.

The need for an alternative source of bone graft is evident and several possibilities have been explored, including bone morphogenetic proteins (BMP) and demineralized bone matrices (DBM). Bone morphogenetic proteins are purely osteoinductive and induce differentiation of pluripotent mesenchymal stem cells into osteoblast and chondrocyte lineages, while DBM serves as an osteoinductive and osteoconductive scaffold.^{12,13} While these graft sources have had varying success rates in the literature, each alternative suffers from a unique complication profile and drawbacks.^{12,14,15} Bone morphogenic protein 2 (BMP-2) has been demonstrated to increase fusion rates by 12% at 24 months. Yet the use of BMP-2 has decreased secondary to substantial cost as well as concerns of increased immediate postoperative pain (odds ratio 1.78 [CI, 1.06 to 2.95]) and cancer rates (relative risk 1.98 [CI, 0.86 to 4.54]) compared to ICBG.¹⁶ Thus, ICBG has remained the gold standard for biologic fusion enhancement in lumbar spine procedures over the years.

Tissue-engineered bone grafts derived from the patient's own cells represent a relatively recent and promising alternative to ICBG.¹⁷ In theory, a tissue-engineered graft provides a bone substitute that is not only osteogenic, osteoinductive, and osteoconductive, but also obviates the need for intraoperative bone harvesting and the associated complications.¹⁸ In the present study, the efficacy of a tissue-engineered bone graft utilizing a decellularized bone scaffold seeded with human hypertrophic chondrocytes differentiated from human adipose derived stem cells is evaluated in an athymic rat spinal fusion model. The tissue-engineered bone graft was constructed based on successful components as shown in literature, with the decellularized bone providing an osteoconductive scaffold with appropriate mechanical properties¹⁹, the clinical potential of adipose derived stem cells²⁰, and the superior bone formation capabilities of differentiated hypertrophic chondrocytes^{21–23}.

Methods

Bone Core Harvest and Decellularization

Bone cores were harvested from bovine juvenile wrists as previously denoted (Figure 1).¹⁹ Cores were cut and measured to a height of 1 cm, diameter of 4 mm, and a density between 0.35 – 0.50 g/mL. Cores were decellularized following published protocols. Briefly, cores were washed in a series of detergent and enzymatic solutions: 1) 0.1% EDTA in PBS for 1 hour, 2) hypotonic buffer consisting of 10mM Tris and 0.1% EDTA in PBS for 12 hours at 4 degrees Celsius, 3) detergent consisting of 10mM Tris and 0.5% SDS in PBS for 24 hours at room temperature on an orbital shaker at 300 revolutions per minute, 4) enzymatic solution of 100 units/ mL DNase and 1 unit/ mL of RNase with 10 mM Tris in PBS at 37 degrees Celsius for 6 hours. After multiple washes in PBS to remove all solutions, the decellularized bone (DCB) cores were frozen and lyophilized.

Stem Cell Isolation, Expansion, and Graft Seeding

Adipose derived stem cells (ADSCs) were obtained from Lonza (Allendale, NJ). The stem nature of the cells was verified by tri-differentiation testing. Cells were expanded until passage 5 in expansion medium consisting of high glucose medium with L-glutamine, 10% fetal bovine serum, and 1% penicillin/ streptomycin. In preparation for seeding, bone cores were disinfected in sterile-filtered 70% ethanol for 2 days, and then incubated in culture medium for 1 day. P5 (ADSCs) were trypsinized, resuspended in culture medium, and then applied to dried scaffolds in a drip method to seed the cores at 30M cells/ mL of scaffold, with a total of 2.5M ADSCs applied to each scaffold. The seeded cores were incubated in expansion medium for 2 days to promote cell attachment and expansion.

Hypertrophic Cultivation and Expansion

After 2 days of expansion medium incubation, the culture conditions were modified to mimic endochondral ossification and produce hypertrophic chondrocytes. With reference to published articles detailing the differentiation of hypertrophic chondrocytes^{22,24} tissue engineered, hypertrophic chondrocyte-based constructs were incubated first in chondrogenic medium (high glucose DMEM (ThermoFisher, Waltham, MA)) supplemented with 100 nM dexamethasone, 50 µg/mL ascorbic acid, 50 µg/ mL proline, 100 µg/mL sodium pyruvate,

1% ITS+, 1% P/S, and 10 ng/mL TGF- β 3) for 2 weeks, followed by 3 weeks of hypertrophic medium (the same as chondrogenic medium, but without TGF- β 3, a dexamethasone concentration of 1 nM, 50 ng/mL of L-thyroxine, and 5mM of β -glycerophosphate). At the culmination of cultivation, grafts were either implanted or harvested for pre-implantation analysis.

Pre-Implantation Cellular Confirmation

After cell cultivation and pre-implantation, a number of grafts were fixed in a 10% formalin solution and decalcified using a 0.5M EDTA solution. Grafts were embedded in paraffin and sectioned in 5 micron slices. Immunohistochemistry and histology were performed on the sections to ensure first chondrogenic differentiation and then hypertrophic maturation. To demonstrate chondrogenic differentiation, deposited GAG was visualized using the typical alcian blue staining protocol widely available. Hypertrophic chondrocyte maturation was demonstrated by collagen type $\times$ immunohistochemistry staining. Staining occurred by first removing ECM components by incubating slides in hyaluronidase (Hyaluronidase Type IV, H3884, Sigma, St. Louis, MO) for 45 minutes, followed by Pronase E (Pronase E, P5147, Sigma, St. Louis, MO) treatment for another 45 minutes. Slides were then blocked with 0.3% hydrogen peroxide in absolute methanol for 30 minutes before following the Vectastain Elite Universal staining kit (Vector Laboratories, Burlingame, CA). Anti-collagen $\times$ antibody was obtained from Abcam (ab49945, Cambridge, MA), and incubated on slides overnight at 4 degrees Celsius. Pre-implantation cellular confirmation of the tissue-engineered graft is shown in Figure 2 by hematoxylin and eosin staining.

Animal Acquisition and Study Design

Thirty-two athymic rats were obtained from an approved vendor and allowed to acclimate in the rodent facility for 2 days in individual cages. All animals were skeletally mature with approximate weight of 300g. The rats were weighed twice per week. If any animal lost >20% weight compared to their pre-surgical body weight, an Institute of Comparative Medicine (ICM) veterinarian was notified. Animals in distress were individually evaluated by the surgical and veterinarian team. A collaborative decision was made regarding continued observation versus euthanasia. To monitor growth and feed intake, each rat was observed at twice daily by ICM technicians and once daily by the surgical team. All protocols were reviewed and approved by the institutional animal care and use committee (IACUC).

The study included two groups with 16 rats in the control group and 15 rats in the experimental group. The control group underwent L4–L5 intertransverse process fusion with surgical decortication and scaffold placement (decellularized bone cores derived from bovine wrists). The experimental group underwent L4–L5 intertransverse process fusion with surgical decortication and tissue engineered scaffold placement (bone cores containing derived hypertrophic chondrocytes). The two time points for rat sacrifice and spine harvest were at 3 weeks and 6 weeks after surgery. Half of the control and seven experimental rats were sacrificed via carbon dioxide inhalation at 3 weeks. The remaining rats were sacrificed at 6 weeks in a similar fashion. Radiographic and histological analysis was performed on rat carcasses after euthanasia at both time points.

Surgical Procedure

In an induction chamber, the rats were anesthetized with inhaled isoflurane (3.0–5.0%) using a controlled vaporizer attached to a scavenging system. Paralube ophthalmic ointment was placed over the rats' eyes to prevent corneal abrasions and desiccation. Buprenorphine SR (1.2 mg/kg) and enrofloxacin (5 mg/kg) were given via a subcutaneous injection. After induction, anesthesia was maintained by inhalation of 2.0% isoflurane using a nose cone ensuring that animals experienced no pain during the procedure. Depth of anesthesia was monitored by the surgeon throughout the procedure and confirmed by lack of pedal reflex and subjective decrease in respiratory rate. Positive toe pinch response or spontaneous movement was an indication for increasing the percentage of inhaled isoflurane. The animals were placed on an electronic warming pad for the duration of the procedure to prevent hypothermia. Vital signs were recorded every 5 minutes perioperatively.

The L4–L5 vertebrae were identified and marked on the skin of the animal. After sterile preparation of the back with povidone-iodine alternating with 70% alcohol, bupivacaine (2–4 mg/kg) was injected subdermally into the surgical site to aid in post-operative pain control. The rat was draped in sterile fashion. Prior to incision, depth of anesthesia was assessed by toe pinch. A 1.5-cm midline incision was made centered on the desired levels, followed by a two incisions in the fascia 3mm lateral to the midline. A paramedian approach was used to expose the transverse processes of L4 and L5.

The transverse processes were decorticated bilaterally with a high-speed burr (Figure 3AB). At this point, a decellularized graft was placed longitudinally across each intertransverse process region in control rats. In experimental rats, the tissue engineered graft containing hypertrophic chondrocytes was placed in similar fashion. The fascia was closed tightly with interrupted suture and skin edges were stapled together.

Radiographic Analysis

All samples were scanned using a Scanco vivaCT 80 system (Scanco Medical AG, Bassersdorf, Switzerland) using energy settings of 55kV and 145μA, and an integration time of 250ms. Images were reconstructed using standard Scanco software at an isotropic voxel size of 31 microns. Grayscale images were subjected to a Gaussian filter (sigma=.8, support=1) to reduce noise and threshold at 30% max grayscale value to produce 3D images. Because the newly-formed bone in the fusion space would naturally be of varying and potentially lower levels of mineralization, grayscale stacks were used for radiographic fusion analysis. Three blinded orthopaedic surgeons familiar with the assessment of spine fusion by μCT independently cycled through the complete stack of images for each specimen, three separate times to obtain inter-observer and intra-observer reliability data for fusion. The spinal fusion was analyzed using three-dimensional interactive viewing and manipulation of the microcomputed tomographic images with VitreaCore software (ViTAL version 6.7.6, A Toshiba Medical Systems Group Company). The quality of fusion was graded on the following predetermined scale defined based on the incorporation of graft with rat transverse processes:

- A. No incorporation

- B. Minimal incorporation at periphery
- C. Central incorporation with interdigitation of graft and rat fusion mass

Histologic Analysis

Upon explantation, grafts were placed in 10% formalin solution for 24 hours. After 0.5M EDTA decalcification and extensive washing, samples were paraffin embedded and sectioned at a 5 micron thickness. Hematoxylin and Eosin (H&E) and Movat's pentachrome staining were performed on the sections.

Statistical Analysis

An independent student t-test was used to assess mean group differences for continuous data. A Chi-squared or Fisher's exact test was used to assess differences in categorical variables (SPSS Version 24; IBM, Armonk, New York). Concordance among raters was determined by the kappa (κ) correlation coefficient. Significance was set at $p < 0.05$.

Results

All rats were assessed for fusion based on the previously described μ CT grading criteria. At three weeks, 5 (62.5%) of the decellularized graft rats fused and 1 (12.5%) had partial fusion, while none of the tissue engineered graft rats had either complete or partial fusion ($p=0.013$). At six weeks, there were 4 (50%) partial fusions but no complete fusions in the tissue engineered graft rats, while 7 (87.5%) of the decellularized graft rats completely fused ($p=0.002$). Among the independent blinded observers, there was excellent inter-observer ($\kappa=0.822$) and intra-observer ($\kappa=0.825$) reliability in grading specimen fusion. Results are summarized in Table 1. μ CT images of decellularized and tissue engineered rats are shown in Figures 4A–C and 5A–C, with 3D reconstructions placed side by side in Figure 6.

Histologic analysis of post-mortem decellularized and tissue engineered rats sacrificed at 6 weeks corroborate the μ CT findings (Figure 7). Bone deposition occurred at the interface of the graft and transverse process in rats fused with decellularized grafts. In rats fused with tissue engineered grafts, non-ossified fibrous tissue was deposited at the transverse process-graft interface.

Discussion

The tissue-engineered scaffold seeded with hypertrophic chondrocytes from human adipose derived stem cells utilized in this study was hypothesized to potentially induce bone formation by mimicking endochondral ossification. This mechanism of action would primarily occur through osteoconductive and osteoinductive pathways. However, this study demonstrated that posterolateral fusion was inhibited in this model.

Decellularized bone grafts, similar to commercially available bone blocks, are highly popular in both the clinical and research environments. As it is the conserved, natural bone matrix stripped of cells, these scaffolds contain native proteins and factors that stimulate bone cell differentiation and bone production.²⁵ The inherent mineral composition and mechanical strength make it a suitable scaffold for immediate clinical implantation into a

defect location, and also a valuable structure from which to develop tissue engineered bone grafts through the differentiation of both osteoblasts and hypertrophic chondrocytes.¹⁹

Theoretically, the inclusion of differentiated hypertrophic chondrocytes in tissue engineered bone grafts should facilitate faster, more complete bone regeneration, as the differentiated cells can respond to the implant environment and deliver cytokines that curb inflammation and promote bone deposition. However, the literature has shown that this benefit is not always realized, as some studies contradict the clear benefit of tissue-engineered grafts²⁶, by demonstrating its inabilities.^{27,28} It appears that in situations in which the implanted graft is connected to the native, decorticated bone with internal fixation and subjected to significant mechanical stress, tissue engineered grafts outperform decellularized grafts. However, a number of situations, including the present study, do not undergo significant mechanical stress, and in these situations,²⁷⁻²⁹ there is no clear difference between the two groups. Hence we theorize that the tissue engineered bone graft in this study may only be viable for primary bone healing with mechanical stress, and not in the currently study environment of secondary bone healing without mechanical stress.

The decellularized grafts are inherently osteoconductive, providing the microarchitecture for bone formation and ultimately bone fusion. In the current study, the transverse processes were decorticated stimulating an osteoinductive and osteogenic release of extensive blood and bone marrow into the implant site. The open pores of the decellularized grafts allowed infiltration of this blood and marrow, delivering important stem cells into close proximity with the influencing decellularized bone matrix. As can be seen in the provided data, this stimulated bone production not only in the pore space of the decellularized graft, but also triggered deposition in the exterior of the scaffold allowing union with the native spine. In contrast, the tissue engineered graft already contained an advanced hypertrophic cartilage matrix within the pore spaces, restricting the blood and marrow presence in the graft interior. As can be seen in the histology, this blockage resulted in a fibrous envelope being produced in the exterior of the graft, preventing new bone formation within the pore space, and restricting union with the native skeleton.

In one of our previous studies, we have demonstrated that the benefit of tissue engineered grafts is the ability to control the regeneration, delaying widespread degradation, and synchronizing bone production with breakdown.²⁶ In the current study, however, it appears that the controlled regeneration dictated by the engineered grafts actually impedes regeneration. The implant site was a positive bone-forming environment, due to the excessive blood and marrow present and lack of destructive mechanical forces, suggesting that control of the degradation and deposition processes was not needed. In contrast, the decellularized grafts deposited significantly more bone, as its osteoinductive nature provided the necessary stimulation.

Lastly, the tissue engineered grafts were not highly suitable for the intended operation. In most studies conducted with tissue engineered grafts, new bone formation only occurs within the defined scaffold that houses the differentiated cells.²⁶ Integration of the graft with the native skeleton occurs when direct contact is maintained between the graft and the native skeleton, allowing the graft to communicate with the native skeleton to outgrow and remodel

the graft. In this study, grafts were placed into the general vicinity of the skeleton, but did not undergo internal fixation with direct contact to the native skeleton. As can be seen in the histology, the grafts did undergo some bone formation and graft turnover, but it exclusively occurred within the initial graft boundaries and did not bridge the distance between the graft and the native skeleton. In contrast, the osteoinductive nature of the decellularized graft can stimulate bone production, and this production is not exclusive to the boundaries of the graft. Therefore, the excessive bone deposition and integration with the native skeleton was observed in the decellularized grafts.

Limitations of this study are similar to other small animal studies in that it is only a surrogate marker for potential bone fusion in humans. Although this study utilized a well-known animal model for posterolateral fusion, which has been described in detail by other groups, the model does not incorporate any internal fixation.^{30,31} However, this particular animal model did allow the use of human hypertrophic chondrocytes without a host vs graft reaction since the rats were athymic. This would not have been possible in a larger animal model. Another limitation is that the quantification of bone production was not possible as this study did not have preoperative μ CT and immediate postoperative scans of the athymic rats, which could have allowed for image subtraction analysis to allow for quantification of bone production. However, this would not have impacted the negative results of this study which clearly demonstrated the human hypertrophic chondrocyte scaffolds inhibited fusion.

In conclusion, the negative results of this study demonstrate that posterolateral fusion at L4/L5 in an athymic rat model was inhibited in rats treated with tissue engineered bone grafts containing differentiated human hypertrophic chondrocytes. This was in stark contrast to the widespread fusion in the decellularized bone grafts. Future research of this type of tissue engineered construct may be better suited for applications in spine surgery involving primary bone healing such as intervertebral fusion. Additionally, different tissue engineered bone strategies are needed for secondary bone healing environments, such as posterolateral fusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The manuscript submitted does not contain information about medical device(s)/drug(s).

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Figure 1.
Bone core harvested from bovine juvenile wrists and cut to a height of 1cm, diameter of 4mm, and density between 0.35–0.50 g/mL.

Figure 2.

Pre-implantation cellular confirmation of tissue-engineered graft. There is a complete absence of cells in the hematoxylin and eosin (H&E) stained decellularized graft, while there are an abundance of cells staining positively for glycosaminoglycans (alcian blue), indicative of differentiated chondrocytes, and type $\times$ collagen, indicative of maturation to hypertrophic chondrocytes. Scale bars: 250 microns.

Figure 3. Intraoperative photograph obtained under the microscope depicting the decortication (A) of a transverse process (black arrow) and insertion (B) of bone graft (*).

Figure 4. Coronal (A), sagittal (B), and axial (C) μ CT images evaluating the decellularized graft integration at 6 weeks. The control rat exhibits robust integration and interdigitation of host bone and the decellularized bone scaffold.

Figure 5. Coronal (A), sagittal (B), and axial (C) μ CT images evaluating tissue engineered graft integration at 6 weeks. There is a complete absence of integration between the tissue engineered graft and rat transverse process at 6 weeks.

Engineered

Decellularized

Figure 6.

Representative three dimensional reconstructions of the implant-transverse process interface for both the decellularized and tissue engineered bone grafts. The reconstructions demonstrate the bone formation and integration of the decellularized graft and the lack of integration in the engineered grafts Scale bars: 1.0 cm.

Figure 7.

Histological analysis of tissue engineered and decellularized bone grafts in rats at 6 weeks. Bone deposition occurred at the interface of the graft and transverse process in rats fused with decellularized grafts. In rats fused with tissue engineered grafts, non-ossified fibrous tissue was deposited at the transverse process-graft interface. B=bone; G=graft; FT=fibrous tissue. Black line demonstrates original implanted graft, indicated the amount of regeneration that occurred in the cylindrical grafts. Scale bars: graft morphology: 2 cm, interface: 500 microns, bone deposition: 50 microns.

Table 1

Comparison of graft integration across control and experimental rats at 3 weeks and 6 weeks.

Time Point	Partial Integration (B)	Complete Integration (C)
3 weeks		
Tissue Engineered	0% (0/7)	0% (0/7)
Decellularized Control	12.5% (1/8)	62.5% (5/8)
6 weeks		
Tissue Engineered	50% (4/8)	0% (0/8)
Decellularized Control	0% (0/8)	87.5% (7/8)