

Bone Marrow–Derived Mesenchymal Stem Cells Enhance Bacterial Clearance and Preserve Bioprosthetic Integrity in a Model of Mesh Infection

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Background: The reported incidence of mesh infection in contaminated operative fields is as high as 30% regardless of the material used. Recently, mesenchymal stem cells (MSCs) have been shown to possess favorable immunomodulatory properties and improve tissue incorporation when seeded onto bioprosthetics. The aim of this study was to evaluate whether seeding noncrosslinked bovine pericardium (Veritas Collagen Matrix) with allogeneic bone marrow–derived MSCs improves infection resistance in vivo after inoculation with *Escherichia coli* (*E. coli*).

Methods: Rat bone marrow–derived MSCs at passage 3 were seeded onto bovine pericardium and cultured for 7 days before implantation. Additional rats ($n = 24$) were implanted subcutaneously with MSC-seeded or unseeded mesh and inoculated with 7×10^5 colony-forming units of *E. coli* or saline before wound closure (group 1, unseeded mesh/saline; group 2, unseeded mesh/*E. coli*; group 3, MSC-seeded mesh/*E. coli*; 8 rats per group). Meshes were explanted at 4 weeks and underwent microbiologic and histologic analyses.

Results: MSC-seeded meshes inoculated with *E. coli* demonstrated superior bacterial clearance and preservation of mesh integrity compared with *E. coli*-inoculated unseeded meshes (87.5% versus 0% clearance; $p = 0.001$). Complete mesh degradation concurrent with abscess formation was observed in 100% of rats in the unseeded/*E. coli* group, which is in contrast to 12.5% of rats in the MSC-seeded/*E. coli* group. Histologic evaluation determined that remodeling characteristics of *E. coli*-inoculated MSC-seeded meshes were similar to those of uninfected meshes 4 weeks after implantation.

Conclusions: Augmenting a bioprosthetic material with stem cells seems to markedly enhance resistance to bacterial infection in vivo and preserve mesh integrity. (*Plast Reconstr Surg Glob Open* 2016;4:e751; doi: 10.1097/GOX.0000000000000765; Published online 17 June 2016.)

Mesh has been used for decades in the diverse field of reconstructive surgery. Despite advances in the development of synthetic and bioprosthetic materials and improvements in surgical techniques, mesh infection remains a challenging and costly complication, particularly in abdominal wall reconstruction.¹ Although mesh placement has reduced the incidence of ventral

hernia recurrence by 50% over suture repair alone,² published rates of infection after this procedure range from 4% to 16%.³ Infection has been reported in as many as 30% of cases after mesh use in contaminated operative fields regardless of the material used.^{4–12} In addition, infection is known to be an independent risk factor for hernia recurrence, and with each procedure, both cost and recurrence risk increase.¹³ An estimated 250,000 ven-

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tral hernia repairs are performed annually in the United States alone,¹⁴ and mesh usage is expected to rise with emerging evidence supporting prophylactic insertion during the index operation.^{15,16}

Implanted bioprosthetic and synthetic materials are thought to resist infection by facilitating neovascularization and host tissue incorporation subsequent to an inflammatory response elicited by the collective trauma of surgery and the presence of a foreign body.^{17–20} To the extent that the scaffold is able to become populated by host cells, it is able to resist and even clear infection. Introduction of an acellular substrate into a surgical wound, however, creates dependence on the host to effect cellular infiltration and immunologic sterilization. This reliance may be problematic in patient populations known to have impediments to wound healing (e.g., because of poorly controlled diabetes, smoking, malnutrition, steroid use, renal failure, and irradiation), especially in the setting of bacterial contamination.

Recently, mesenchymal stem cells (MSCs) seeded onto the surface of bioprosthetics have been found to enhance mesh incorporation into surrounding host tissue, increase neovascularization, and improve mechanical properties of meshes.^{21–26} MSCs have been shown to benefit wound healing not only by promoting tissue regeneration and increasing the recruitment of macrophages and endothelial cells into the wound but also by possessing immunomodulatory and antimicrobial activities.^{27–29} Animal studies have determined that the administration of MSCs in a variety of in vivo conditions augments antibacterial responses against both Gram-negative and Gram-positive pathogens, decreases inflammation, and leads to faster bacterial clearance.^{28–32} To date, no published studies have addressed whether seeding mesh materials with MSCs improves the host response to bacterial contamination of implanted materials. The objective of this study was, therefore, to determine whether seeding a commercially available bioprosthetic with allogeneic bone marrow-derived MSCs improves infection resistance in vivo after inoculation with a common Gram-negative pathogen.

MATERIALS AND METHODS

Animals

A total of 26 male Sprague-Dawley rats weighing approximately 350 g were used. Animals were obtained from Taconic (Germantown, N.Y.) and housed in the Tripler Army Medical Center animal facility. The study protocol was approved by the Institutional Animal Care and Use Committee at the Tripler Army Medical Center. Investigators complied with the policies as prescribed in the US Department of Agriculture Animal Welfare Act and the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Study Design

An established rat infection model^{13–35} was used to evaluate whether seeding acellular, noncrosslinked bovine

pericardium (Veritas Collagen Matrix, Synovis Life Technologies Inc., St. Paul, Minn.) with MSCs improves resistance to bacterial contamination after inoculation with *Escherichia coli* (*E. coli*). Twenty-four rats underwent subcutaneous implantation in the dorsum with two 3.75-cm² pieces of mesh. Rats were assigned randomly to 1 of the 3 groups, with 8 rats per group. Group 1 was implanted with unseeded mesh and received 200 μ L of sterile saline into the surgical wound after mesh placement but before skin closure. Group 2 was implanted with unseeded mesh and received 200 μ L of bacterial suspension containing 7×10^5 colony-forming units (cfu) *E. coli* to simulate a contaminated surgical field. Group 3 was implanted with allogeneic rat bone marrow-derived MSC-seeded mesh and received 200 μ L of bacterial suspension containing 7×10^5 cfu *E. coli*. Veritas Collagen Matrix was selected because its use is described for the reconstruction of complex abdominal wall defects.³⁶ Four weeks after surgery, explants underwent microbiologic and histologic analyses. This time point was chosen because we wished to examine a period during which meshes should be actively remodeling and acute surgical wounds should have healed.

Isolation of Rat Bone Marrow-Derived MSCs and Preparation of MSC-Seeded Mesh

After lethal intraperitoneal injection of a pentobarbital-based euthanasia solution, bone marrow from 2 additional rats was mechanically harvested by flushing femurs and tibias with Medium 199 (M199E, Sigma Aldrich, St. Louis, Mo.) using a 22-gauge needle and 3-mL syringe under aseptic conditions. The pooled cell suspension was passed through a 40- μ m strainer, centrifuged at 200g at 4°C for 5 minutes, resuspended in 25 mL of cell culture medium (MesenCult Proliferation Kit with MesenPure, STEMCELL Technologies Inc., Vancouver, British Columbia, Canada), and cultured on two 75-cm² tissue culture flasks at 37°C and 5% CO₂. MSCs were expanded in vitro in a complete medium (M199E/10% fetal bovine serum [MSC-qualified fetal bovine serum, Life Technologies Corp., Grand Island, N.Y.]/1 \times penicillin–streptomycin). To demonstrate the multipotency of expanded cells used for seeding, aliquots of MSCs at passage 5 were successfully differentiated into adipocytes and osteoblasts using MesenCult adipogenic and osteogenic differentiation media from STEMCELL Technologies Inc. according to the manufacturer's instructions (not shown).

Seven days before implantation, Veritas Collagen Matrix was cut into 2.5 \times 1.5 cm strips using an aseptic technique. Meshes were presoaked in MSC-qualified FBS for approximately 1 hour to facilitate cell adhesion and then placed into 6-well culture plates (1 mesh per well) containing a complete medium. Using a pipette, 7.5×10^5 MSCs at passage 3 were dripped onto each mesh (equivalent to 2×10^5 MSCs per cm²), and plates were incubated at 37°C and 5% CO₂. On day 3, meshes were flipped over and seeded with an additional 7.5×10^5 MSCs, yielding a total of 1.5×10^6 MSCs per mesh (4×10^5 MSCs per cm²). This number of cells is consistent with seeded cell densities used in previous studies.^{21,23–26} Cells were given a fresh medium every 2 to 3 days and were implanted in

rats after 7 days in culture. This incubation period is consistent with preimplantation seeding protocols advanced by others.^{22–24} On the day of surgery, excellent cell viability (>95% estimated semiquantitatively) and adherence were visualized on 4 meshes (not implanted in rats) using an Olympus IX71 fluorescent microscope (Olympus America Inc., Center Valley, Pa.) as determined by a LIVE/DEAD cell viability assay (Life Technologies Corp.) following the manufacturer's instructions (Fig. 1). Before evaluation, samples were moved to a new 6-well plate to ensure that visualized cells were adherent to meshes and not to the bottom of wells.

Bacterial Inoculum Preparation

E. coli was chosen as the contaminant for this study given its clinical relevance as a common enteric organism and based on previous work in our laboratory, demonstrating profound mesh degradation and clinically apparent abscess formation after *E. coli* colonization on noncrosslinked porcine dermis.³³ *E. coli* (ATCC #25922) was obtained from American Type Culture Collection

(Manassas, Va.). Two days before surgery, an aliquot was thawed from frozen stock and cultured on blood agar plates for 48 hours with a minimum of 1 passage between plates. Culture concentration was determined by spectrophotometry (optical density₆₀₀) and compared with a predetermined growth curve. Cultures were brought to the desired concentration in 0.9% sterile saline and verified by plating serial 10-fold dilutions (in triplicate) of the final solution used during surgery.

Surgery and Tissue Collection

Surgery, anesthesia, and analgesia were performed as described previously.³³ Briefly, bilateral 3-cm dorsal incisions were made 1 cm lateral to the spine. A subcutaneous pocket was created at each incision site, and 1 piece of mesh (of the same type and inoculum) was placed into each pocket. The bacterial inoculum (200 μ L suspension of 7×10^5 cfu *E. coli*) or sterile saline (200 μ L) was pipetted onto each implanted mesh before skin closure with sterile stainless steel clips (Braintree Scientific, Braintree, Mass.).

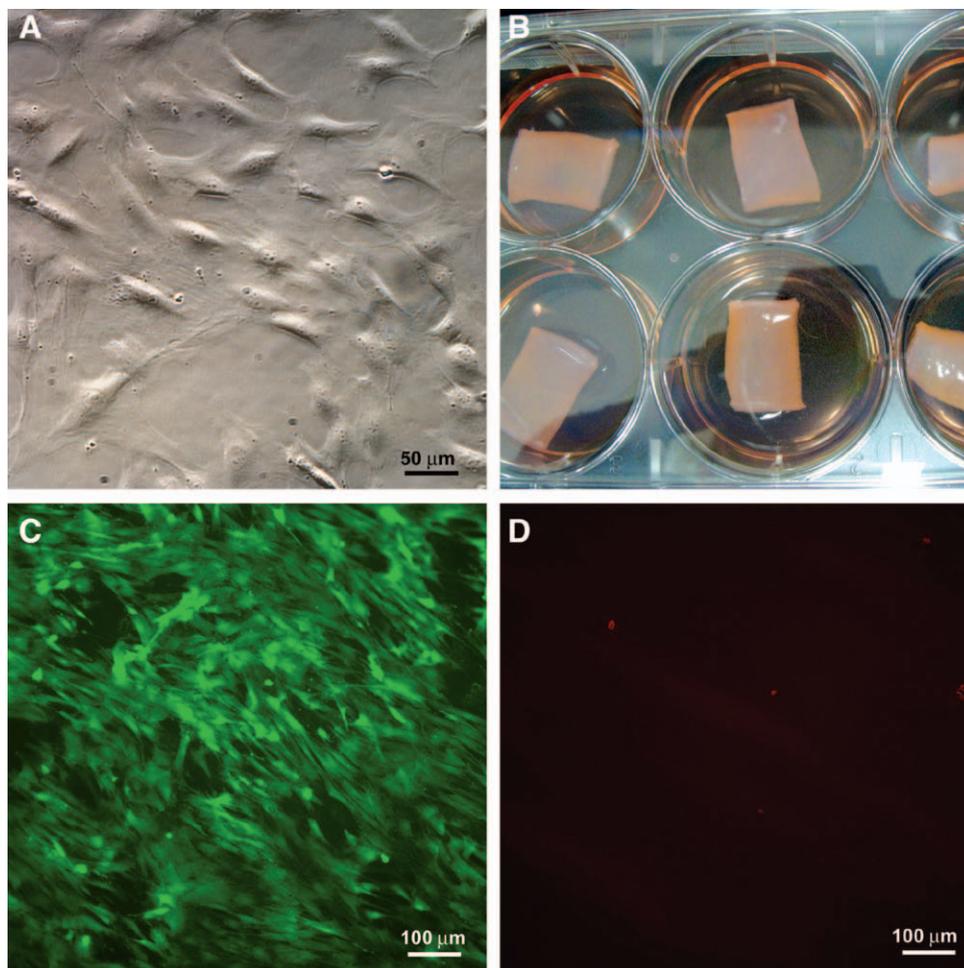


Fig. 1. Veritas Collagen Matrix seeded with rat bone marrow–derived MSCs. MSCs at passage 3 in culture (A), MSC-seeded meshes on day 6 after seeding 7.5×10^5 cells per side (equivalent to 2×10^5 MSCs per cm^2 per side) (B), and representative images of a LIVE/DEAD cell viability assay performed on MSC-seeded mesh after 7 days in culture stained with calcein to view live cells (green; C) and ethidium to view dead cells (red; D). Note the high cell viability and density on mesh at 7 days.

Animals were evaluated daily for signs of local infection, sepsis, pain or distress, or wound complications.

On postoperative day 28, rats were deeply anesthetized using a combination of ketamine (75 mg/kg) and dexmedetomidine (250 µg/kg) administered intraperitoneally, and cardiac puncture was performed to determine blood-stream infection rates as described.³³ Rats were killed by intracardiac injection of a pentobarbital-based euthanasia solution, and meshes were carefully excised under sterile conditions. In the event that the mesh could not be readily identified, the subcutaneous space was explored from the dorsal midline to the anterior axillary line to rule out migration and to confirm complete degradation. The length and width of each explanted mesh were measured to evaluate contraction (decrease in surface area) of the implant. One strip of mesh from each animal was divided into 2 equal pieces for bacterial recovery and histologic analyses. The second implant (if present) was stored at -80°C for future biomechanical testing.

Bacterial Recovery at Explant

Explanted meshes were submerged in 1 mL 0.9% sterile saline and vortexed for 1 minute to dissociate adherent bacteria as described.³³ Serial 10-fold dilutions were plated in triplicate on blood agar and incubated at 37°C for 24 hours before counting colonies. Gram stains were performed, and meshes were scored as positive if the clinical isolates were Gram negative. Bacterial clearance was defined as the number of animals with sterile cultures divided by the total number of inoculated animals and expressed as a percentage.

Histology

Samples were fixed in formalin and embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin. Specimens were evaluated by 2 blinded investigators at $40\times$ to $200\times$ magnification. They were graded for cellular infiltration, cell types present, extracellular matrix deposition, scaffold degradation, fibrous encapsulation, and neovascularization according to a scale used by Jenkins et al³⁷ and used previously by our laboratory.¹⁷ Higher scores on this scale represent more favorable implant remodeling characteristics. A composite histologic score was also calculated for each sample by taking the average of each of the scores in each of the subcategories.

Statistical Analysis

All results were reported as mean \pm SEM. The Kruskal–Wallis 1 way analysis of variance on ranks was used to determine weight differences among the 3 experimental groups followed by pairwise multiple comparisons using the Dunn's method to identify specific differences between groups. Comparisons between 2 groups were performed using the *t* test or nonparametric Mann–Whitney rank sum test if indicated. Proportions pertaining to bacterial clearance, wound complications, and biologic mesh degradation comparisons were performed using the Fisher exact test. Statistical analyses were performed using SigmaPlot 11.2 software (Systat Software Inc., San Jose, Calif.) with a *p* value less than 0.05 considered significant.

RESULTS

Postoperative Course

All animals survived the 4-week postoperative period. During the first week, 1 rat in the *E. coli*/unseeded mesh group had reduced eating habits and activity level compared with other rats in this group likely because of complications from anesthesia, and therefore, this animal was conservatively removed from weight analyses to prevent confounding. Because activity and weight returned to normal during the second half of the study period, this animal was used in all subsequent analyses. Significant differences were noted in weight gain among the 3 groups during the first 2 postoperative weeks ($p < 0.002$, week 1; $p < 0.007$, week 2; Fig. 2). Rats implanted with *E. coli*/unseeded mesh demonstrated reduced weight gain ($p < 0.05$) relative to the other 2 groups 1 week after surgery. At week 2, both *E. coli* groups demonstrated reduced weight gain ($p < 0.05$) compared with the saline/unseeded mesh group, but differences disappeared by week 3.

Wound complications, including abscess formation and skin breakdown, differed among the 3 groups. Seroma and hematoma formation were not observed in any animal. Although there were no wound complications in the saline group, 100% of rats that were implanted with *E. coli*/unseeded mesh developed bilateral abscesses (8/8 rats; 16/16 meshes) and 50% of these animals (4/8 rats) developed skin breakdown. In contrast, only 1 rat (12.5%) in the *E. coli*/MSC-seeded group developed a unilateral abscess (1/8 animals; 1/16 meshes), and no animal in this group developed skin breakdown. Thus, abscess formation was markedly reduced in animals implanted with *E. coli*-inoculated MSC-seeded mesh compared with *E. coli*-inoculated unseeded mesh ($p < 0.001$). Seeding meshes with MSCs was also protective for skin breakdown, but this did not reach statistical significance ($p = 0.08$).

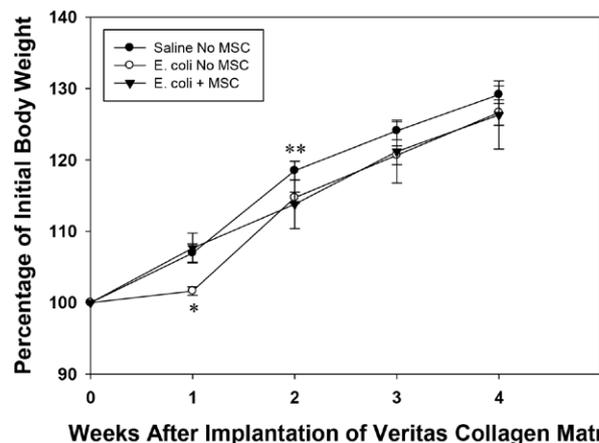


Fig. 2. Rat weights after implantation of MSC-seeded and MSC-unseeded Veritas Collagen Matrix. One week after surgery, animals implanted with *E. coli*-inoculated MSC-seeded mesh demonstrated improved weight gain relative to animals implanted with *E. coli*-inoculated unseeded mesh. Weight differences among groups disappeared by week 3. * $p < 0.05$, *E. coli*-inoculated unseeded mesh versus the other 2 groups; ** $p < 0.05$, saline versus the 2 *E. coli* groups.

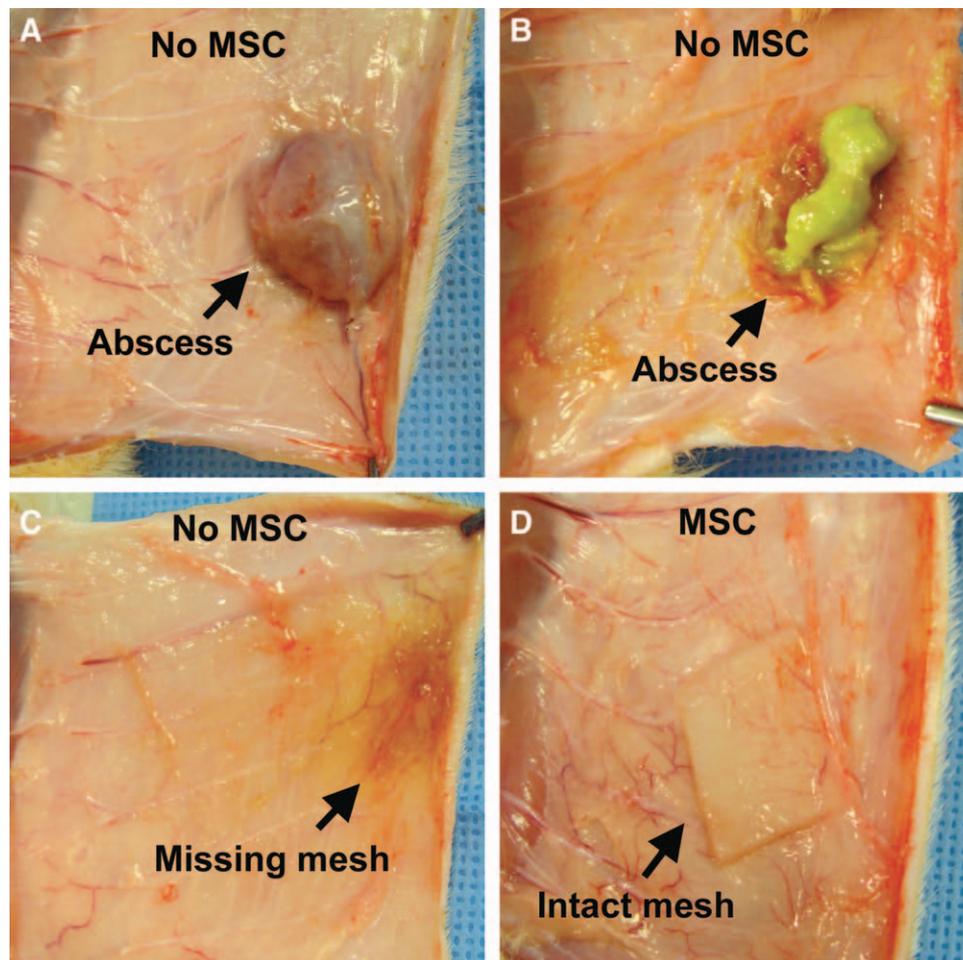


Fig. 3. Fate of *E. coli*-infected Veritas Collagen Matrix 4 weeks after implantation. Abscess formation (A and B) and complete degradation (C) of unseeded meshes contrasts with the preserved structure of MSC-seeded meshes (D) in animals inoculated with *E. coli*.

Macroscopic Findings

At necropsy, all unseeded meshes inoculated with saline demonstrated minimal incorporation into the surrounding host tissue and were easily removed with minimal adhesiolysis. In contrast, no mesh material was identified in any animal in the *E. coli*/unseeded mesh group (0/16 meshes; Figs. 3 and 4). The infections that developed at every implantation site in all animals in this group seemed to effect complete mesh degradation. In many cases, abscesses were still present at the time of harvest. Remarkably, 15 of 16 meshes (93.8%) in the *E. coli*/MSC-seeded mesh group were recovered intact ($p < 0.001$) with the lone exception occurring in the animal that developed a unilateral abscess. These MSC-seeded meshes demonstrated greater adherence to surrounding tissue compared with unseeded meshes inoculated with saline and required more extensive dissection to facilitate removal. Recovered meshes in the saline/unseeded group ($n = 16$) underwent a $49\% \pm 0.5\%$ reduction in surface area, whereas recovered meshes in the *E. coli*/MSC-seeded group ($n = 15$) exhibited a $46 \pm 2\%$ reduction ($p = 0.19$).

Microbiologic Findings

Quantitative cultures at 4 weeks revealed the presence of viable *E. coli* on 1 of 8 (12.5%) MSC-seeded meshes inoculated with *E. coli*, and this sample had a bacterial load of only 33 cfu per mesh. None of the saline/unseeded meshes were positive. Although no mesh material was recovered in the *E. coli*/unseeded mesh group, 2 samples of excised tissue at the site of implantation underwent microbiological testing and had bacterial loads of 2.2×10^7 and 2.7×10^7 cfu per sample. These results equate to 87.5% bacterial clearance in the MSC-seeded group versus 0% in the unseeded group ($p = 0.001$) in *E. coli*-inoculated animals. No rats had positive blood cultures.

Microscopic Findings

Representative images and histologic scores are presented in Figs. 5 and 6, respectively. Notably, scores could not be obtained for the *E. coli*/unseeded group because all implanted meshes in this group were completely degraded, which was confirmed on histologic examination (Fig. 5). At 4 weeks after implantation, saline/unseeded meshes and *E. coli*/MSC-seeded meshes were



Fig. 4. Representative Veritas Collagen Matrix explants 4 weeks after implantation. Unseeded meshes inoculated with saline exhibited minimal host tissue incorporation (A), unseeded meshes inoculated with *E. coli* exhibited abscess formation and complete mesh degradation (B), and MSC-seeded meshes inoculated with *E. coli* exhibited preserved mesh integrity, increased adherence to surrounding host tissue, and improved bacterial clearance compared with unseeded infected meshes (C).

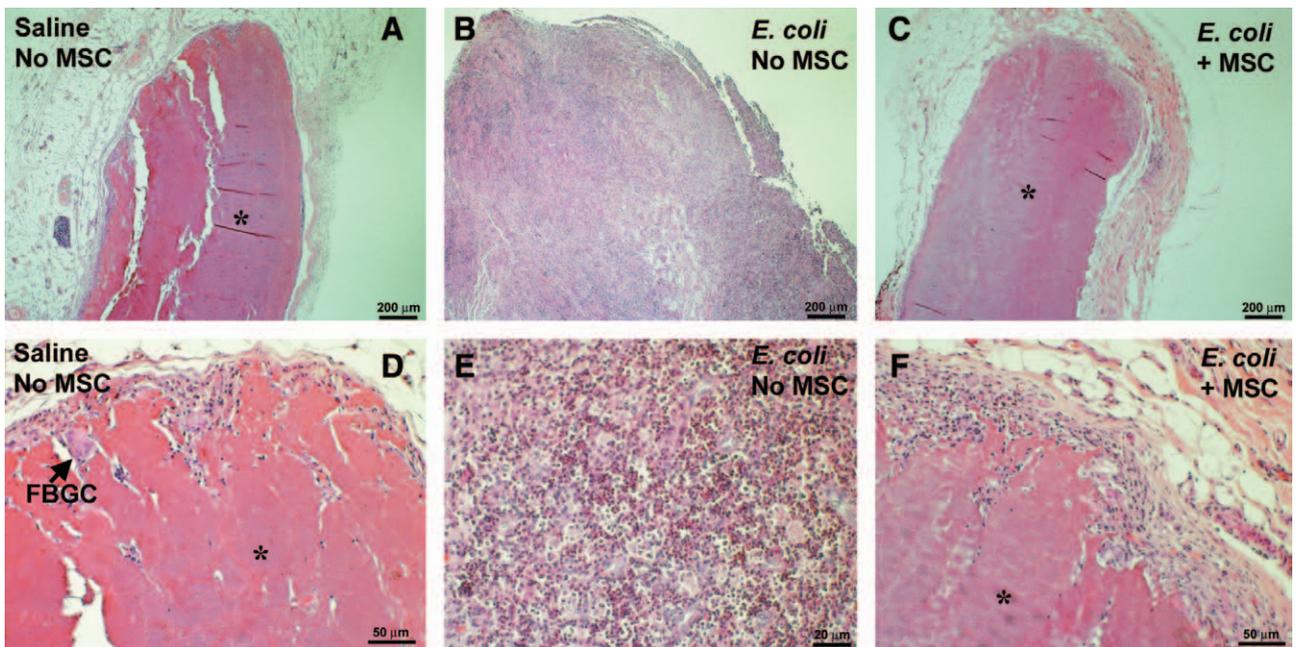


Fig. 5. Representative hematoxylin and eosin-stained sections of unseeded mesh inoculated with saline (A and D), completely degraded unseeded mesh inoculated with *E. coli* (B and E), and MSC-seeded mesh inoculated with *E. coli* (C and F). Infiltration of cells and blood vessels can be visualized near the periphery of intact implants (*) and in the newly deposited connective tissue surrounding the implants. In *E. coli*-inoculated animals, unseeded meshes exhibited severe acute inflammation with no evidence of remaining mesh material (E), whereas MSC-seeded meshes remained intact and demonstrated mild inflammation (F) similar to unseeded meshes inoculated with saline. FBGC, foreign body giant cell.

well tolerated and exhibited minimal acute inflammation, with inflammatory cells consisting predominantly of mononuclear cells and few foreign body giant cells (Fig. 5). Cellular infiltration and tissue remodeling were generally limited to the periphery of implants. Statistically significant differences were not observed for any of the histological parameters between *E. coli*/MSC-seeded and saline/unseeded meshes (Fig. 6).

DISCUSSION

The ideal mesh material is one that enables optimal integration into host tissue, provides long-term structural integrity, and resists bacterial colonization; however, the search for the ideal mesh continues.¹ Although acellular bioprosthetic materials have been developed in an effort

to improve biocompatibility and resistance to infection compared with synthetic meshes, experimental and clinical data suggest that in actuality, they may not resist infection or improve outcomes when used in contaminated surgical fields.^{33,38} Findings from this study revealed that the augmentation of a bioprosthetic (Veritas Collagen Matrix) with MSCs markedly improves bacterial clearance in vivo and enables preservation of mesh integrity when contaminated with *E. coli*.

Although it is possible that Veritas Collagen Matrix exhibits increased sensitivity to degradation in the setting of bacterial contamination, previous work from our laboratory and others have described similar findings in bioprosthetic materials ranging from acellular porcine dermis (Strattice, LifeCell Corp., Branchburg, N.J.)³³ to acellular human dermis (AlloDerm, LifeCell Corp) and

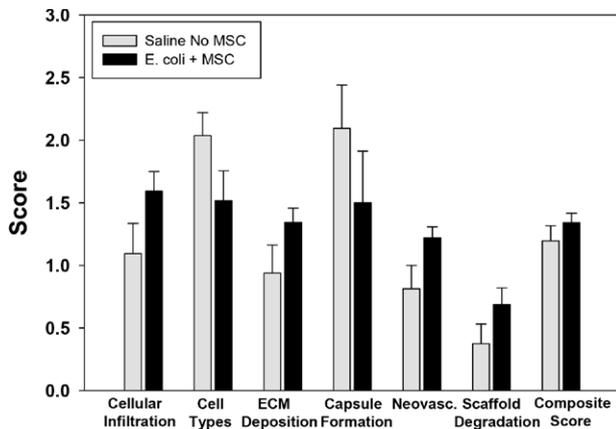


Fig. 6. Histologic scoring of unseeded meshes inoculated with saline (n = 8) and MSC-seeded meshes inoculated with *E. coli* (n = 8) 4 weeks after implantation. Scores could not be obtained for unseeded meshes inoculated with *E. coli* because all implanted meshes in this group were completely degraded. A composite histologic score was calculated by taking the average of the scores in each of the 6 subcategories. Higher scores represent more favorable remodeling characteristics. Values are reported as mean \pm SEM. No statistically significant differences in histological parameters were found between the 2 groups. ECM, extracellular matrix; Neovasc., neovascularization.

porcine small intestinal submucosa (SIS, Surgisis Biodesign, Cook Biotech, Bloomington, Ind.).^{34,35} This may be a result of enzymatic degradation by the invasive pathogens themselves or caused by collagenases and matrix metalloproteinases produced by infiltrating activated leukocytes during an increased inflammatory response. The consequences of early, aberrant mesh degradation and subsequent mesh failure in the setting of a reconstructive procedure are difficult and costly to remediate. Because the presence of MSCs on the surface of a bioprosthetic protects against degradation by promoting effective bacterial clearance and downregulation of the inflammatory cascade,^{28–32} cellularized meshes may provide a promising option for use in contaminated surgical fields. Additional benefits of MSC-seeded implants include enhanced incorporation into surrounding host tissue, improved mechanical properties, and increased neovascularization.^{21–26} The macroscopic demonstration of improved gross tissue incorporation in the MSC-seeded group certainly lends additional support to this growing body of literature.

Several studies have begun to investigate the immunomodulatory and antimicrobial properties of MSCs.^{30–32,39,40} Human MSCs have been shown to cause direct bacterial killing by secreting antimicrobial peptides, such as the human cathelicidin hCAP-18/LL-37, which is effective against both Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria.³² Other antimicrobial proteins, such as β -defensin-2, lipocalin 2, and keratinocyte growth factor, also have been shown to mediate antibacterial effects of MSCs.^{31,39,40} In an animal model of sepsis, MSC administration enhanced bacterial clearance, in part, because of increased phagocytic activity of host immune cells.³⁰ In addition, MSC administration reduced inflammation, downregulated

inflammation-related genes, and upregulated genes involved in promoting antigen presentation, phagocytosis, and bacterial killing.³⁰ Thus, MSCs seem to have the ability to modulate the acute phase response while enhancing phagocytosis and upregulating expression of antimicrobial peptides. This is likely responsible for reducing the negative consequences of unchecked inflammation while directly enhancing pathogen clearance.^{27,30}

Although the direct clinical relevance of this study may prompt speculation, taking cellular materials from bench to bedside is not an abstraction. Human viable wound matrices, such as Grafix (Osiris Therapeutics Inc., Columbia, Md.), which contains nonimmunogenic, cryopreserved MSCs, are already in use. This viable matrix shows promise in wound healing with particular emphasis on reducing rates of infection.⁴¹ Given the favorable characteristics of MSC-containing materials and the size/profitability of this market, one may envision clinical availability of a hybrid mesh (e.g., a viable wound matrix grafted to a biologic or synthetic substrate) or a preseeded cryopreserved mesh for reconstructive procedures in the not-too-distant future.

Limitations of this study exist. MSCs were not labeled before seeding meshes, and therefore, identification of MSCs was not attempted in tissues 4 weeks after implantation. In addition, this investigation did not quantify clinical outcomes other than infection and was restricted to a monomicrobial inoculum using a single bioprosthetic material. Preclinical studies examining the benefits of various MSC-seeded bioprosthetics for reconstructive procedures, such as abdominal wall repair in the setting of polymicrobial contamination, are warranted.

CONCLUSIONS

Augmenting a bioprosthetic mesh with MSCs seems to markedly enhance its resistance to infection, preserve mesh integrity, and facilitate incorporation into surrounding host tissue. Although others have demonstrated increased collagen deposition and neovascularization in seeded bioprosthetic materials, this study is the first to our knowledge revealing improved bacterial clearance in vivo.

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