Evaluation of a Biocomposite Mesh Modified with Decellularized Human Amniotic Membrane for Intraperitoneal Onlay Mesh Repair

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ABSTRACT: Various materials and approaches have been used to optimize the biocompatibility of mesh to reduce the implant-induced host response in intraperitoneal onlay mesh (IPOM) repair. Ineffective host integration, limited resistance to contamination, and untargeted administration hinder the wider application of the currently available clinical options. In this study, human amniotic membrane (HAM) was decellularized, fully characterized, and compared with porcine small intestinal submucosa (SIS) in terms of its structure, components, and bioactivity. In an in vivo study, HAM was reinforced with silk fibroin (SF) membrane, which was fabricated as a biodegradable submicroscale template by electrospinning, to construct a bilayer composite mesh. The independent SF membrane, associated with HAM and SIS, was evaluated for tissue remodeling in vitro. The HAM–SF and SIS meshes were then characterized morphologically and implanted intraperitoneally into Sprague–Dawley rats for 28 days for macroscopic investigation of their integration into the host via interactions of regulatory factors. After decellularization, HAM formed a bioagent-rich collagen-based acellular structure. HAM was superior to SIS in concurrently suppressing the expression of transforming growth factor β1 (TGF-β1) and proangiogenic proliferation. When HAM, SF, and SIS were used as regenerative scaffolds, they showed qualified biocompatibility, cell infiltration, and degradation in vitro. Comparatively, macroscopic observation after implantation indicated that HAM–SF induced less-intensive intraperitoneal adhesion and weaker inflammatory responses at the interface but greater angiogenesis in the explant than SIS. Analysis of the expression of regulatory factors showed a greater quantity of hepatocyte growth factor (HGF) in HAM, which partly inhibited the expression of TGF-β1 and promoted vascular endothelial growth factor (VEGF)-induced angiogenesis. This bioactive interaction appeared to be responsible for the better host integration, making HAM more biocompatible than SIS in IPOM repair. When combined with SF, HAM displayed similar mechanical properties to SIS. In conclusion, HAM displayed better bioactivity and biocompatibility than SIS. After its reinforcement with SF, HAM–SF is a promising biocomposite mesh for IPOM repair.

1. INTRODUCTION

The intraperitoneal onlay mesh (IPOM) technique has been used extensively in ventral hernia repair to restore the abdominal wall structure with sufficient mechanical support. The development of laparoscopic techniques has markedly alleviated intra-abdominal harassment during IPOM, reducing the complication rate. Whereas meshes used in certain condition induce acute or chronic immune responses, inflammation, and adhesion because they are foreign bodies, and this remains an unsolved problem. Researchers have continued to optimize the meshes used for intraperitoneal application to minimize the risks of these common post-operative complications. Current clinically feasible strategies, including using a sheetlike barrier such as expanded poly(tetrafluoroethylene) (ePTFE), or degradable compounds, such as hyaluronic acid gel, aim to provide a smooth interface to shield the injured peritoneum and mesh from the viscera. However, undegradable ePTFE is associated with poor host integration and may cause mesh shrinkage, migration, infection, and even explantation, while the efficiency and endurance of hyaluronic acid gel, which is considered a rapidly degraded partial protector, remains controversial. Various innovative approaches have recently been explored, including nanoparticle coating, drug release, and stem cell loading, and appear to be promising techniques for modifying off-the-shelf meshes. However, in terms of their clinical applicability, they fall far short of standards for contamination resistance with full degradation, targeting ability, safety, cost-effectiveness, and handling. Based on these prerequisites, a modified, naturally
derived mesh with inherent biocompatibility and that offers appropriate mechanical support is an admissible alternative. 10

Allografts of human amniotic membrane (HAM), an avascular collagen matrix in the innermost layer of the placenta, have anti-inflammatory, anti-fibrotic, and antiadhesive properties and accelerate wound healing. Because the extracellular matrix (ECM) contains cytokines and growth factors and displays low immunogenicity after decellularization, the HAM possesses excellent biocompatibility and has been used as a tissue-engineering template in various fields. 11-13 Although the exact mechanism of the remodeling behavior of HAM remains unclear, it is thought to be partly mediated by the downregulation of transforming growth factor β1 (TGF-β1) in the TGF-β1/SMAD signaling pathway. 14 With this modulation, host cells play essential roles in synthesizing collagen and establishing the dynamic balance between the restoration and overdeposition of the ECM, which can be followed by adhesion. Several studies of hernia repair have reported that an HAM-integrated polypropylene mesh reduced adhesion during intraperitoneal repair. 5,15,16 However, the polypropylene may cause long-term complications after the degradation of HAM. HAM is expected to be superior to prosthetic mesh in avoiding adhesion and to modify the application of polypropylene interperitoneally depending on its inherent properties. But whether it is more favorable than clinical biological meshes for IPOM repair remains unclear. Moreover, the mechanism of bioactive action is not well understood because the diversity of structures and bioagents present depends upon the preparation of the HAM.

To overcome these limitations of HAM-based mesh in intraperitoneal applications, electrospun silk fibroin (SF) was introduced instead of polypropylene to reinforce the HAM. SF, a biodegradable polymer derived from silkworm cocoons, can be fabricated into an ECM-mimicking structure with electrospinning and has been shown to be a good engineered candidate for tissue regeneration. 17 In the scenario, the bioactivity from HAM and reinforced mechanical supports from SF were integrated in the HAM–SF biocomposite mesh, which was evaluated and compared with a biological mesh after its intraperitoneal placement. Decellularized porcine small intestinal submucosa (SIS) mesh is a clinically available biological mesh used for IPOM repair that is prepared from the porcine jejunum by removing the host cells, after which it retains its three-dimensional, collagen-rich, bioagent-containing ECM structure. 18 It was selected as the control in our study. Because their decellularized ECM structures are similar, the structure and components of HAM were first compared to those of SIS. A macroporous electrospin SF membrane was then prepared as a strengthening support. The cyocompatibility, bioactivity, cell infiltration, and degradation of HAM and SF were compared to those of SIS before their direct contact with the parietal and visceral peritoneum in IPOM repair. The differences between HAM and SIS were compared to clarify the relevance of the structures and components to their behavior in vitro. The HAM–SF mesh was compared with the SIS mesh after they were implanted in Sprague–Dawley rats for 28 days in terms of the effect of repair, host response, integration, and mechanical performance. The expression of bioactive regulatory factors by the explants was also measured to investigate the interactions and coordination of the processes involved. The aims of this work were to determine the suitability of an innovative biocomposite mesh for IPOM repair and to gain an insight into the interactions of the bioactive components affecting the integration of HAM with the host tissues.

2. RESULTS

2.1. Decellularization and Characterization. To visualize decellularization, HAM and SIS were histologically characterized with hematoxylin and eosin (H&E) and Masson’s trichrome staining (Figure 1A,B). The residual DNA was quantified and compared (C, D). The structures were characterized with transmission electron microscopy (E, F). Collagen I and elastin were stained and compared between samples (G, H). Data are represented as mean ± standard deviation, n = 3, *P < 0.05.

Figure 1. After decellularization, HAM and SIS were evaluated with hematoxylin and eosin (H&E) and Masson’s trichrome staining (A, B). The residual DNA was quantified and compared (C, D). The structures were characterized with transmission electron microscopy (E, F). Collagen I and elastin were stained and compared between samples (G, H). Data are represented as mean ± standard deviation, n = 3, *P < 0.05.
layer, fibroblast layer, and spongy layer, leaving only the acellular basal layer and the compact layer, which were composed of loose collagenous fibrils and had a thickness of 20–35 μm. The thickness of the denser fibrous structure of SIS was 60–95 μm (Figure 1E,F). The two-photon microscopy images clearly confirmed the distribution of collagen I and elastin fibrils, which were mainly localized in the matrices and dominated the mechanical properties of the membranes. They showed a relatively finer fibrous structure in HAM, but bundles of fibers were aggregated in SIS (Figure 1G). Consistent with this, quantitative analysis confirmed that there was more collagen I but less elastin in SIS than in HAM (Figure 1H).

2.2. Chemical Analysis of Components. The total proteins and regulatory growth factors at detectable levels in HAM and SIS were analyzed comparatively and are listed in Table 1. According to the quantity of hydroxyproline produced, SIS had more than 2 times more total protein than HAM (P < 0.05). Of the growth factors identified, the amounts of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were much higher in HAM than in SIS (P < 0.05), whereas TGF-β1, tumor necrosis factor α (TNF-α), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and platelet-derived growth factor BB (PDGF-BB) were maintained at similar levels (P > 0.05). Intriguingly, there was no detectable hepatocyte growth factor (HGF) in SIS, but high quantities in HAM (P < 0.05), which may reflect the differences in specific signaling molecules in the dynamic host incorporation.

2.3. Preparation and Characterization of Biocomposite Meshes. 2.3.1. Preparation and Structure. An overview of the prepared samples indicated that HAM had a more transparent and homogeneous appearance than SIS, and SF was fabricated into a macroporous prototype with a pore diameter of 2.0 mm (Figure 2A). Representative scanning electron microscopy (SEM) images also showed that both HAM and SIS had similar smooth, dense surfaces on the epithelial/mucosal side, with loose and porous networks on the stromal side. SF had an ECM-mimicking nanostructure. The electrospun SF had smaller pores (8.0 ± 3.2 μm) and lower porosity (46 ± 10%) with a more homogeneous structure (P < 0.05; Figure 2F). After integration, the compact HAM–SF was 86.0 ± 5.4 μm thick, with no obvious delamination in cross section. The corresponding value for the diversiform SIS was 88.0 ± 5.3 μm (P > 0.05; Figure 2G,H).

2.3.2. Biocompatibility and Bioactivity. The biocompatibility of the meshes was determined in terms of cellular adhesion and proliferation after incubation for 72 h. SEM images showed spindled-shaped fibroblasts with stretched-out pseudopods scattered on the surfaces of SF. A confluent monolayer of cells formed on and covered SIS and presented a more well-oriented appearance than those on HAM (Figure 3A1–A5). The skeleton of human dermal fibroblasts (HDFs) stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin confirmed the cell-friendly microstructures of the various scaffolds (Figure 3B1–B5). The results of the cell counting kit-8 (CCK-8) assay indicated that there were no

![Figure 2](https://dx.doi.org/10.1021/acsomega.9b03866) Overview of the prepared HAM, SF, and SIS (A). Representative SEM images show the structures of HAM and SIS, on both the epithelial/mucosa and serosal sides, associated with electrospun SF (B–D). Scale bar = 20 μm in (B)–(D), including for the inserted images. The electrospun SF was analyzed by FTIR spectroscopy before and after treatment (E). The pore sizes and porosity of the scaffolds were measured independently (F). Cross sections of HAM–SF and SIS (G, H).

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**Table 1. Components of Total Collagen and Varied Detectable Growth Factors in HAM and SIS**

| Component  | HAM                       | SIS                      |
|------------|---------------------------|--------------------------|
| total collagen | (370.5 ± 40.0) × 10^3     | (860.2 ± 108.6) × 10^3   |
| VEGF       | 10.7 ± 1.2*               | 1.91 ± 0.4               |
| TGF-β1     | 9.0 ± 2.6                 | 14.5 ± 4.0               |
| bFGF       | 31.65 ± 13.30*            | 16.05 ± 6.10             |
| PDGF-BB    | 30.9 ± 12.8               | 36.6 ± 12.7              |
| HGF        | 120.6 ± 42.8*             |                         |
| TNF-α      | 6.15 ± 1.8                | 5.24 ± 1.2               |
| EGF        | 4.5 ± 1.1                 | 3.0 ± 1.0                |
| IGF-1      | 6.54 ± 1.5                | 6.36 ± 1.4               |
| IL-6       | 4.95 ± 0.9                | 3.29 ± 0.7               |

*n = 5, *P < 0.05.*
significant differences in cell viability between the HAM and SIS samples, which was similar to the cell viability on tissue culture plate (TCP) ($P > 0.05$). However, the value for SF was lower than that for SIS ($P < 0.05$; Figure 3C).

Under similar conditions, the expression of TGF-$\beta$1 by the HDFs on HAM and SIS was detected with immunofluorescent staining (Figure 3E). Compared to the stained cells on TCP, the HDFs on HAM and SIS both displayed weaker fluorescence, although the cells on SIS showed greater fluorescence than those on HAM. In the quantitative analysis (Figure 3E), the amount of TGF-$\beta$1 secreted by the cells on HAM was $7.5 \pm 1.7 \text{ pg/\mu g}$, which was approximately half as much as that secreted on SIS ($13.0 \pm 2.2 \text{ pg/\mu g}$, $P < 0.05$). In contrast, the highest concentration of TGF-$\beta$1 in all of the groups was secreted by the cells on TCP ($21.5 \pm 2.5 \text{ pg/\mu g}$, $P < 0.05$).

The bioactive features of the meshes were also evaluated based on the morphology, immunofluorescence, and proliferation of the HUVECs on the various scaffolds. SEM images showed a fully confluent layer of adherent HUVECs on HAM, whereas the cells on SIS formed an almost confluent monolayer. Following the interactions with the scaffolds, HUVECs secreted rounded matrices. However, there were fewer HUVECs scattered on SF (Figure 3F1−F3). CD31 staining confirmed that HAM promoted greater CD31 expression than SF or SIS, providing a microenvironment for exquisite endothelialization and potential vascularization. Compared to SIS, SF showed inferior bioactive CD31 expression, which was attributed to the properties of the biopolymer after electrospinning and subsequent treatment (Figure 3G1−G3). This was also reflected in the level of HUVEC proliferation on scaffolds at different density (Figure 3H). Consistent with this, HAM showed the highest proliferate value of all of the groups ($P < 0.05$). The behavior of SIS was statistically similar to that of TCP ($P > 0.05$), whereas SF showed a relatively weaker performance ($P < 0.05$).

2.3.3. Cell Infiltration and Enzyme Degradation. An infiltration test was performed to evaluate the remodeling potential of the scaffolds (Figure 3I1−I3). On day 7, all of the scaffolds presented biocompatible interfaces being completely covered with a monolayer of smooth muscle cells (SMCs). By day 28, the cross sections of the scaffolds confirmed different

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**Figure 3.** HDFs were seeded on different scaffolds, and their morphology, immunofluorescence, and proliferation were evaluated after incubation for 72 h (A−C). Scale bar = 200 $\mu$m in (A) and (B). TGF-$\beta$1 expression was detected and quantified in terms of the total protein (D, E). Scale bar = 50 $\mu$m in (D1)−(D3). The morphology, immunofluorescence, and proliferation of human umbilical vein endothelial cells (HUVECs) that proliferated on the different scaffolds were evaluated after incubation for 7 days (F−H). Scale bar = 200 $\mu$m in (F1)−(F3). Scale bar = 50 $\mu$m in (G1)−(G3). Cell infiltration on the scaffold surfaces was characterized with SEM; and 4′,6-diamidino-2-phenylindole (DAPI) staining (bright white) of the cross sections using laser confocal scanning microscopy (H1−H3). Scale bar = 100 $\mu$m in (H1)−(H3). The degradation of the various scaffolds was calculated and compared (I). Data are presented as mean ± standard deviations in (C), (E), (H), and (J); n = 3, $^*P < 0.05$ in (C), (E), and (H). $^*P < 0.05$ vs HAM in (J).
Figure 4. Macroscopic images of the intraperitoneal sides of the meshes from the HAM−SF and SIS groups after implantation for 28 days (A−C). The white arrows indicate the organs adhered to explants. The explants were retrieved to detect the partial peritoneal integration (D1−D3). The dashed boxes show the sites of induced adhesion. The tenacity and surface areas of the adhesions were analyzed according to the criteria described in Section 5.6 (E). The changes in the thickness of the meshes were measured (F). Data are presented as mean ± standard deviations in (E) and (F); n = 3, *P < 0.05.

Figure 5. Host responses to the explants were analyzed with H&E staining, and matrix incorporation was analyzed with Masson’s trichrome staining (A−D). Scale bar = 100 μm in (A)−(D). Collagen I and fibronectin were detected at the sites of adhesion with immunostaining (E−H). Scale bar = 50 μm in (E)−(H). The inflammatory responses, represented by CD54 and CD68, were detected with immunohistochemical staining (I−L). The total areas of positive staining were quantified as percentages (Q, R). Angiogenesis was detected in the HAM−SF and SIS explants with CD31 immunostaining and analyzed as the percentage mean area of vascularity (M−P, S). Scale bar = 100 μm in (I)−(P). Data are presented as mean ± standard deviation in (Q), (R), and (S); n = 3, *P < 0.05.
degrees of infiltration of distinct nuclei-stained cells. Although the electrospun scaffold was originally developed to retard cell infiltration in nanoscale structures, the SF fabricated under our conditions had a submicroscale structure that allowed cell penetration after static incubation for 28 days.

Enzymatic degradation was tested to evaluate the biodegradability of the scaffolds, especially of the recrystallized electrospun SF, before their implantation (Figure 3J). After the first week, all of the scaffolds showed relatively rapid degradation, with no significant difference in the weight lost (P > 0.05). Thereafter, the scaffolds changed at a much slower rate, as the soluble components decreased. By the end of week 4, the residual weight of HAM (62.0 ± 2.8%) had decreased significantly more than that of SIS (70 ± 2.5%, P < 0.05) in response to the nature of the ECM, which was a loose structure in HAM that tended to collapse after losing its integrity. SF degraded more slowly, consistent with the response of the polymer to aqueous treatment (78.0 ± 2.2%, P < 0.05).

2.4. Macroscopic Observation. Macroscopic observation at 28 days after surgery showed that there were no apparent signs of hematomas, microabscess, bulge, or herniation on the abdominal wall side in any of the 10 rats in the HAM–SF or SIS groups. Only one rat in the HAM group displayed mild seroma (Figure 4A,B). After laparotomy, observation of the peritoneal side revealed that one of the HAM-implanted rats had developed an adhesion of the cecum to the abdominal wall, while severe adhesive agglutination of the colon and greater omentum was found in two rats in the SIS group (Figure 4C1–C4). After resection of the abdominal wall, an overview of the granulating tissue in immunostaining were performed to detect the host response than HAM.

Indirect evidence that SIS elicited more aggressive reactions was the residual weight of HAM (62.0 ± 2.8%) had decreased significantly more than that of SIS (70 ± 2.5%, P < 0.05) in response to the nature of the ECM, which was a loose structure in HAM that tended to collapse after losing its integrity. SF degraded more slowly, consistent with the response of the polymer to aqueous treatment (78.0 ± 2.2%, P < 0.05).

2.5. Histological Analysis. Histological staining and immunostaining were performed to detect the hist response and the integration of the mesh 28 days after implantation.

2.5.1. Adhesion Formation. Representative images of H&E-stained tissues confirmed that SIS formed adhesions of dense granulating tissue infiltrated by mononuclear inflammatory cells, whereas HAM showed a slightly thickened sublayer with matrix deposition and cell infiltration in the various stages of remodeling (Figure 5A,B). Masson’s trichrome staining showed that HAM contained more regularly arranged collagen than SIS. However, when SF was integrated into the mesh, HAM–SF showed less incorporation than SIS (Figure 5C,D).

The explants were also stained to detect the expression of collagen I and fibronectin at the interface, at which the predominant adhesions tended to form. The images confirmed that more collagen I and fibronectin were deposited at the adhesive interface of SIS, whereas the expression in HAM was fairly low (Figure 5E–H).

2.5.2. Inflammatory Response. Immunohistochemical staining was used to quantify the inflammatory response to the explants (Figure 5I–L,Q,R). The images of CD54 expression showed rare leukocyte infiltration in the HAM and SIS groups after 30 days. There was no difference in the positive staining percentage between the two groups (0.50 ± 0.10 vs 0.70 ± 0.20%, respectively; P > 0.05).

Macrophages were detected by immunostaining for CD68, which confirmed that they were mainly located on the connected interfaces of the explants. The SIS explants showed more positive staining for CD68 than the HAM explants (5.30 ± 0.90 vs 2.53 ± 0.50%, respectively; P < 0.05), indicating that they induced a stronger host immune response and more inflammation-induced fibrosis.

2.5.3. Angiogenesis. When angiogenesis was detected (Figure 5M–P,S), the HAM explants showed notably greater CD31 immunofluorescence and had formed mature vascularized networks, whereas the SIS explants contained fewer branched vessels that were loosely connected. The HAM explants showed greater percentage vascularity (8.99 ± 1.02%) than the SIS explants (5.17 ± 0.90%; P < 0.05).

2.6. Regulatory Factors. To estimate the potential interactions and coordination of the regulatory factors involved in the integration of the explants, we identified and quantified those that participated fully in the host response and the integration process. TGF-β1-positive staining was much weaker in the HAM explants than in the SIS explants. Conversely, more pronounced HGF staining was observed in the HAM explants (Figure 6A–D). Consistent with this,

Figure 6. Explants were analyzed with immunostaining for TGF-β1 and HGF (A–D). The proteins were also quantified with western blotting (E). In the peritoneal fluid, inflammatory cytokines represented by TNF-α and interleukin 6 (IL-6) were measured with enzyme-linked immunosorbent assays (ELISAs) (F, G). Scale bar = 50 μm in (A)–(D). Data are expressed as mean ± standard deviation in (F)–(G); n = 3. *P < 0.05.

Western blotting revealed faint expression of TGF-β1 but the prominent expression of HGF in HAM (Figure 6E), combining with the opposite trends in SIS, confirming that the inhibition of TGF-β1 was at least partly mediated by the high quantity of HGF in HAM.

The inflammatory regulators in the explants were measured in terms of the concentrations of TNF-α and IL-6 in the peritoneal fluid. The concentrations of TNF-α (110.0 ± 20.8 pg/mL) and IL-6 (21.0 ± 13.3 pg/mL) were lower in the
HAM group than in the SIS group (270.0 ± 30.2 and 69.0 ± 21.2 pg/mL, respectively; P < 0.05), indicating that intra-peritoneal inflammation was milder in the HAM group (Figure 6F,G).

2.7. Mechanical Properties. The mechanical properties of the HAM–SF implants before and after their integration were measured and compared to those of the SIS implants in vitro. The maximum load, tensile strength, and Young’s modulus were similar between HAM and SF and between HAM–SF and SIS (P > 0.05, Figure 7A–D). With respect to the above mechanical terms, we found a comparative tendency in their performances. Before integration, both HAM and SF clearly showed less mechanical support than SIS (P < 0.05), but it increased to a level similar to that of SIS after integration (P > 0.05). Stiffness was greatest for SF (5.03 ± 1.6 N/mm) than for the other materials tested (Figure 7D, P < 0.05), implying that SF was less compliant as the intra-abdominal pressure fluctuated, which profoundly affected the HAM–SF. Integrating SF with HAM compromised the stiffness of HAM from 0.93 ± 0.03 to 3.90 ± 0.40 N/mm and ultimately exceeding that of the SIS mesh (2.54 ± 0.26 N/mm; P < 0.05). The values of the explants on the tensile strength test, which measured the failure at the interface between the mesh and the host abdominal wall, reflect the strength of the integrated abdominal wall. With the rapid degradation of ECM after implantation, they all underwent some extent of dropping when limited incorporation formed initially. After remodeling for 28 days, the mechanical support of the explants was not significantly lower than that of the implants. The strength terms of maximum load, tensile strength, and Young’s modulus in stabilizing this overlapping area did not differ between HAM–SF and SIS, although the HAM explants were stiffer (P < 0.05).

3. DISCUSSION
The human amniotic membrane is an avascular membrane with a thickness of 35–60 μm. It has been used in a variety of surgical strategies to promote epithelialization and tissue remodeling, as well as to prevent tissue adhesion, with a low inflammatory response. However, the bioactive features of HAM are dependent on a diversity of preparation techniques, and no consensus on these has been reached or conflicts resolved. Other than their intrinsic characteristics, various preparatory processes, including denudation, deepithelialization, and decellularization, together with dehydration or frozen storage and various irradiation techniques, determine the bioactivity of HAM. In this study, the epithelial layer, fibroblast layer, and spongy layer were fully removed by the decellularization of HAM to minimize its immunogenicity and antigenicity, which is considered to be a “double-edged sword” leading to the loss of bioactive modules and the intact structure simultaneously. In this study, we confirmed that HAM maintained an acellular, collagen-abundant structure with thin, loosely arranged fibrils compared to SIS histologically and morphically. In the analysis of the ECM components, collagen I of HAM, which provides the primary fibrous skeleton for the amnion by mainly localizing to the basement membrane and compact layer, was looser in appearance than in the structure of SIS. Accordingly, finer elastin fibers remained in the HAM as the determinants of membrane’s compliance, providing resilience and elasticity to the scaffold. Western blotting confirmed that there was less collagen I but more elastin subunits in HAM than in SIS. These findings, combined with the lower proportion of total collagen in HAM, may explain why the mechanical properties of HAM were inferior to those of SIS, which justified the subsequent introduction of electrospun SF to HAM.

HAM and SIS showed the expected biocompatibility naturally, which is an essential precondition for their intraperitoneal application. After enzymatic and irradiation treatments, a wide variety of growth factors still remained in the HAM that are known to regulate host response in a coordinated manner. One of these bioagents, TGF-β1, is a multifunctional growth factor that is probably responsible for the clinical benefits of HAM, including its antifibrosis and anti-inflammatory and antiadhesion properties, through down-regulating the expression of its receptor. Although similar amounts of TGF-β1 were detected in HAM and SIS in this study, as for other soluble signals, such as TNF-α, EGF, IGF-1, and PDGF-BB, higher quantities of TGF-β1-mediated factors were detected in HAM, including HGF, VEGF, and bFGF. HGF is a therapeutic agent that antagonizes the profibrotic action of TGF-β1, and it appeared to be abundant in HAM but undetectable in SIS, which is capable of modulating the bioactivity of HAM.

In the preparation of the SF membrane, aqueous electrospinning and post-treatment with water vapor were used to improve its biocompatibility by avoiding the use of organic solvents, resulting in the biocompatible interface to serve as the reinforced outer surface of the biocomposite mesh. Because the biopolymer structure was prepared without stimulating agents, the bioactivity of SF was inferior to that of SIS or HAM, as expected. The weaker staining of TGF-β1 in the HDFs cultured on HAM confirmed the TGF-β1-suppressing property of HAM. In contrast, HAM contained much more VEGF and bFGF than SIS, indicating its superiority in promoting endothelial cell proliferation, migration, and potential angiogenesis. Furthermore, the practical benefits of HAM may also be amplified because HGF enhances VEGF-induced angiogenesis. Although the complex interacting signaling pathways involved were not clarified in this study, the
accelerated proliferation of HUVECs on HAM, with elevated CD31 expression, supports our speculations and provides a clue to the exact mechanism involved.\textsuperscript{33}

The pore size, porosity, and degradation of the structure of HAM, SF and SIS were examined, especially because the electrospun nanoscale structure of SF used to limit cellular infiltration.\textsuperscript{34} With the water vapor treatments, the SF was transformed from an α-helix and random coil conformation to a partly stable β-sheet structure, avoiding the full conformation induced by methanol/ethanol and following inactive degradation. We showed that the SF scaffold degraded by approximately 22% within 4 weeks. By using the SF membrane with 2.0 mm porous structure, we intend to mimic the macroporous mesh used clinically, aim to provide sufficient mechanical support and promote the cell infiltration following sufficient host integration, and avoid forming adhesion simultaneously.\textsuperscript{35} We confirmed the SMC infiltrated through pores of about 10 μm in diameter. Similar results were observed when cells were grown on electrospun mats with an average pore size of 8 μm.\textsuperscript{36,37} HAM and SIS had comparatively heterogeneous and bigger pore sizes, facilitating cell infiltration and rapid degradation.

Macroscopic observation showed that the postoperative performance of the HAM–SF group was better than that of the SIS group, presenting with timely parietal peritoneal recovery, a moderate host response, and only slight intra-peritoneal adhesions. After the intraperitoneal implantation of the mesh, the success of repair mainly depends on the dynamic processes of host integration, including cell infiltration, neoangiogenesis, ECM restoration/deposition, and the formation of neoperitoneum, which were subsequently interpreted historically.

In this study, the different integration processes of HAM–SF and SIS were attributed to the interactions of the bioagents involved. With CD31 staining, we showed that the dominant angiogenic factors in HAM included VEGF, bFGF, and HGF. Kwon et al. reported that combined treatment with HGF and VEGF was superior to treatment with either factor alone in enhancing therapeutic angiogenesis while avoiding inflammation.\textsuperscript{38} This coordinated effect was confirmed in the HAM explants. HAM also induced more HGF-stained cells with lower TGF-β1 expression, together with less intraperitoneal adhesion and inflammation compared to the SIS explants. HGF stimulates the proliferation and migration of various cell types by phosphorylating tyrosine in c-Met. The mechanism is considered to partly involve the prevention of peritoneal thickening by HGF and downregulation of TGF-β1 and collagen I. This inhibits intraperitoneal adhesion by regulating IFN-γ and plasminogen activator inhibitor-1, which play essential roles in maintaining the reciprocal balance with tissue plasminogen activator.\textsuperscript{39} In the comparison of HAM explants with SIS explants on regulating TGF-β1, SF membrane actually participated in the process. The result indicates that SF has not reversed the superiority of HAM to SIS on inhibiting TGF-β1 and on antiadhesion. To testify whether SF enhances the suppression of TGF-β1 or not in the process, the expression of TGF-β1 on the abdominal side of mesh and the exploration of its mechanism need to be further evaluated. The faint immunohistochemical staining of CD54 and CD68, associated with the low concentrations of IL-6 and TNF-α in the peritoneal fluid, confirmed the anti-inflammatory function of HAM. In addition to inducing growth factors, HAM exerts its anti-inflammatory activity by entrapping inflammatory cells for apoptosis, reducing the expression of proinflammatory cytokines, and enhancing the secretion of anti-inflammatory cytokines, such as IL-4, IL-10, and IL-1α, glycosaminoglycan, and matrix metalloproteinases, some of which were not investigated in this study.\textsuperscript{40,41}

With the 28 day remodeling, the mechanical support afforded by the HAM–SF explant was similar to that of SIS statistically. It was consistent with our previous study that the tensile strength of non-cross-linked ECM-abundant scaffold usually reached a nadir 2 weeks following implantation and reinforced from 4 weeks.\textsuperscript{42} At this point, the mechanical support of the HAM explant, in terms of its tensile strength and Young’s modulus, slightly exceeded that of SIS, which was reversed in vitro. This implies that the subsistent support of the slow-remodeling SF and the host integration of HAM together contributed to ensure its excellent performance. The electrospun SF membrane that was fabricated as a macroporous prototype with bio-compatible interface also promoted the integration with the abdominal wall and increased the stiffness of the explant. In our scenario, the two-layer HAM–SF was designed to have a similar thickness and mechanical strength to the one-layer SIS, to optimize the comparative analysis of their bioactivity. To match the strength of the natural abdominal wall, the mechanical properties of HAM–SF could be increased by using a multilayered structure in future studies.

It is well known that implants are essential for IPOM repair. Despite extensive efforts to identify the bioactivity of naturally derived meshes, we could not draw any definite conclusions. The host integration of bio-implants depends on many factors, including bioactive modules, dose-dependent interactions, ECM components, and targeted cell responses in specific pathways, for example, and these still remain largely unclear. In the comparisons with SIS in this study, HAM was shown to suppress the TGF-β1- and VEGF-induced properties of the host tissue, probably mediated by HGF, which sheds light on the exact interactions involved. The specifically designed SF was introduced to construct a bilayer HAM–SF mesh. Our in vivo results confirm that the composite mesh has the bioactive features of HAM and is reinforced by SF, facilitating IPOM repair with antiadhesive, anti-inflammatory, and proangiogenic effects. Our attempt to construct a biocomposite mesh for intraperitoneal hernia repair extends the possible applications of HAM. However, further studies are required to explore and fully characterize the mechanism(s) involved.

4. CONCLUSIONS

In this study, we prepared a biocomposite mesh by integrating decellularized HAM with electrospun SF. This novel structure takes advantage of the superior bioactivity of HAM and the reinforced mechanical support of SF, and it is therefore preferable to SIS for IPOM repair. The preponderance of various bioagents in HAM was shown to be responsible for its benefits in terms of the host responses and mesh integration.

5. EXPERIMENTAL SECTION

5.1. Materials. Bombyx mori silkworm cocoons were obtained from the Second Silk Company (Zhejiang, China). Recombinant human vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), and hepatocyte growth factor (HGF) quantikine ELISA kits were obtained from R&D Systems (Minneapolis, MN). Human angiogenesis ELISA strip I and II for profiling eight cytokines
kits were obtained from Signosis, Inc. (Santa Clara, CA). Cell counting kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Mouse monoclonal anti-CD31 antibody, fluorescein isothiocyanate (FITC)-conjugated phalloidin, rabbit anti-collagen I, and mouse anti-elastin antibodies with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse polyclonal anti-TGF-β1, anti-CD68, and anti-CD54 antibodies and the bicinechonic acid (BCA) protein assay kit were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Dulbecco’s modified Eagle’s medium (DMEM), endothelial cell growth basal medium-2 (EBM-2), fetal bovine serum (FBS), 0.25% trypsin (DMEM), endothelial cell growth basal medium-2 (EBM-2), fetal bovine serum (FBS), 0.25% trypsin—ethylenediaminetetraacetic acid (EDTA), and other supplements for cell culture were obtained from Invitrogen Corp. (Carlsbad, CA).

5.2. Decellularization and Characterization. To obtain the optimal HAM, the donors were selected based on the following requirements: (i) pregnancy period of at least 35 weeks; (ii) no malformation of the baby; (iii) screened during pregnancy for Hepatitis B virus, Hepatitis C virus, Human Immunodeficiency virus, human T-cell lymphotropic virus type 1, and syphilis. Placentas were obtained from five donors, who had undergone cesarean section, with prior informed consent. All procurement procedures were approved by the Institutional Review Boards at both Shanghai Tongji University School of Medicine (Shanghai, China) and the Affiliated Shanghai East Hospital (Shanghai, China). In the decellularization process, the HAM was separated from its underlying chorionic membrane by blunt dissection and then rinsed with phosphate-buffered saline (PBS) and immersed in 1% Triton X-100 with agitation at 100 rpm for 24 h to remove the spongy layer of the amniotic tissue. The HAM was then treated with 0.25% trypsin + 0.02% EDTA for 4 h at 37 °C to completely remove the epithelial monolayer and fibroblast layer, including the host cells. Residual nucleic acid sequences were removed by incubation in 50 U/mL DNase (Sigma, St. Louis, MO) in Tris base at 37 °C for 2 h. After lyophilization, the HAM was cut into 5 × 5 cm² pieces for packaging. The decellularized SIS product was prepared using a process described previously. All samples were sterilized with γ-rays (30 kGy, 60Co; Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai, China) and stored at −80 °C before use.

For the histological analysis, the HAM and SIS monolayers were stained with hematoxylin and eosin (H&E) and modified Masson’s trichrome to confirm decellularization and collagen preservation, respectively. The ECM structure was characterized in detail with transmission electron microscopy (Carl Zeiss GmbH, Oberkochen, Germany). To quantify DNA, round pieces (10 mm diameter) were punched from the samples and digested with 250 pg/mL papain (Sigma) at 60 °C for 4 h. The DNA was then extracted and collected from the mixtures according to a purification protocol and quantified with a Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher, Waltham, MA).

The distribution and quantity of collagen I and elastin, which typically dominate the mechanical behavior of the natural ECM, were visualized by two-photon laser scanning microscopy (Fluoview FV1000 MPE, Olympus, Tokyo, Japan) and analyzed by western blotting (ChemoCam Imager 8.3, Intas), respectively. For immunostaining, a rabbit anti-collagen I antibody (diluted 1:100; Sigma) and labeled with Alex Fluor 488 (1:400; Sigma) was used for primary staining, and a mouse anti-elastin antibody (1:100; Sigma) labeled with Alex Fluor 594 (1:400; Sigma) was used for secondary staining. For the western blots, the samples were extracted with 5 M guanidinium chloride containing 0.02% (w/v) NaNO₃ (pH 7.0) for 24 h. The insoluble residues were incubated for 48 h at 37 °C with a similar enzyme digestion process for further extraction. All of the collected proteins were dialyzed, purified, lyophilized, and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred to membranes, the membranes were incubated with antibodies directed against GAPDH (diluted 1:1000, Sigma), collagen I, and elastin (diluted 1:1000; Sigma) at 4 °C overnight and then with horseradish-peroxidase-conjugated secondary antibodies (diluted 1:2000; Cell Signaling Technology, Europe) for 1 h at room temperature. The immunoblotted protein bands were observed using a Prime enhanced chemiluminescence kit (Sigma, St. Louis, MO).

5.3. Chemical Analysis of Components. The dominant collagens and growth factors that are the major components of the ECM were quantified to explain the bioactive merits of HAM and SIS. To measure the total collagen in the materials, micronized samples were digested in 125 μg/mL papain with acid hydrolysis and reacted with chloramine T solution (Mallinckrodt Baker, Phillipsburg, NJ), followed by an incubation in p-dimethylaminobenzaldehyde (Sigma-Aldrich) to determine the quantity of hydroxyproline present (pg/mg), which reflects the amount of total collagen when multiplied by a constant of 7.25. To quantify the various regulatory growth factors contained in HAM and SIS, the samples were cut into small pieces and ground in liquid nitrogen. The micronized samples were weighed, mixed (100 mg tissue/mL) with proteinase inhibitors dissolved in ice-cold lysis buffer (Promega, Madison, WI), and gently rotated at 4 °C for 48 h. The extracts were clarified by centrifugation at 12 000g for 30 min at 4 °C and quantified with specific single factor (R&D Systems) and multiplex ELISA kits (Signosis).

5.4. Preparation and Characterization of Biocomposite Membrane. 5.4.1. Preparation of Electrospun Silk Fibroin Membrane. The SF was first extracted from B. mori cocoons as previously described. In this study, a 30 wt% aqueous SF solution was used for electrospinning. The process was as follows: 30 × 30 cm² nonwoven, porous fabric was wrapped around a panel to collect the nanofibers. The SF solution was loaded into a 2 mL syringe and electrospun through a blunt stainless steel needle (outer diameter, 0.8 mm). The flow rate was optimized to remain at 0.4 mL/h with a syringe pump (KD Scientific, Holliston, MA) at a voltage of 15 kV (VC100, Glass Mann, Japan). A distance of 12 cm was maintained between the needle tip and the collector. The room temperature and humidity were maintained at 25–28 °C and >60%, respectively, during the whole process. The collected membranes were treated with water vapor at 70 °C for 60 min in vacuum to stabilize the structure for further characterization. The membranes were also analyzed by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy to investigate the changes in the secondary structure of SF after treatment. The FTIR data were gathered on a Nicolet 5700 spectrometer (Thermo Fisher Scientific). Each spectrum was acquired in the transmittance mode by the accumulation
of 256 scans at a resolution of 2 cm$^{-1}$ and a spectral range of 4000–400 cm$^{-1}$.

5.4.2. Preparation and Characterization of Mesh. To prepare the bilayer structure, an 8 wt% SF gel was used as a water–solvent binder to combine the electrospun SF with HAM, with the stromal side positioned outward. The composite was immersed in PBS at 4 °C for 2 h and then lyophilized. An SIS mesh of the same size was also prepared as the control for comparison. The morphology of the HAM and SF layers in the composite mesh showed an integrated cross section, and they were characterized and compared to those of SIS by scanning electron microscopy (SEM; ESEM XL 30, Philips, Eindhoven, the Netherlands). Examination of the porosity and pore size of the independent layers of five samples was conducted using a mercury porosimetry device (AutoPor-eV, Micromeritics, GA) under a tightly controlled pressure.

5.4.3. Biocompatibility. To determine the biocompatibility of the scaffolds, 1.0 cm$^2$ samples were punched and preconditioned with 1 mL of DMEM for 2 h before cell seeding. Human dermal fibroblasts (HDFs, 50 μL) were cultured in DMEM containing 10% FBS, 1% 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C under 5% CO$_2$ and then seeded on the scaffolds in 24-well plates at a density of 1 × 10$^4$ cells per well. After the cells were allowed to attach for 2 h, 450 μL of medium was added. The cell-seeded scaffolds were transferred into new 24-well plates and cultured for another 72 h, during which time the medium was not refreshed. The attachment of the HDFs to the various scaffolds was observed with SEM, and the cytoskeletons of the HDFs were stained with phalloidin–FITC (diluted 1:50; Invitrogen Corp.) and observed by confocal laser scanning microscopy (LSM 710, Carl Zeiss GmbH). HDF proliferation was detected with CCK-8 (Dojindo), and the absorbance of the cells was measured at a wavelength of 450 nm (BioTek Instruments, Inc., Winooski, VT). HDFs seeded on a polystyrene tissue culture plate (TCP) were used as a positive control.

5.4.4. Bioactivity. Under the conditions used in the biocompatibility test, the HDFs cultured on HAM or SIS were immunostained to determine TGF-β1 production. In brief, the scaffolds were fixed, blocked, and then incubated overnight with an anti-TGF-β1 antibody (diluted 1:50; Santa Cruz Biotechnology) at 4 °C and then with an Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (diluted 1:400; Sigma) for 2 h at room temperature. Cell nuclei were stained with Hoechst 33258 (0.5 mg/mL; Sigma). The supernatant (150 μL) of the culture medium was collected from each well. The actual amount of TGF-β1 secreted by the HDFs was calculated by measuring the amount in the culture medium with an ELISA kit (R&D Systems). The total cellular protein from the different scaffolds after lysis was measured by a BCA protein assay kit (Santa Cruz Biotechnology). The concentration of TGF-β1 protein in the culture supernatant was normalized to the corresponding total cellular protein content to accommodate differences in the number of cultured cells.

The bioactive features of angiogenesis were evaluated by seeding the various scaffolds with human umbilical vein endothelial cells (HUVECs). HUVECs were cultured in EBM-2 containing 5% FBS and 1% 100 U/mL penicillin and 100 units/mL streptomycin at 37 °C under 5% CO$_2$. Aliquots (50 μL) of HUVECs were seeded in 24-well plates at a density of 1 × 10$^5$ or 5 × 10$^5$ cells per well. After cell attachment, 450 μL of medium was added and the cells were incubated at 37 °C. The medium was refreshed every 3 days. After proliferation for 7 days, the cells were morphologically characterized. CD31 expression was verified with an anti-CD31 antibody (diluted 1:50; Sigma), an Alexa-Fluor-594-conjugated goat anti-mouse IgG antibody (diluted 1:400; Sigma), and Hoechst 33258 labeling. To measure the cell metabolic activities at different densities, a CCK-8 assay was performed after 7 days to compare the cells with the control cells seeded on TCP.

5.4.5. Cell Infiltration and Enzyme Degradation. To detect the cellular infiltration of the scaffolds in vitro, human umbilical vein smooth muscle cells (SMCs) were seeded on the membranes (1.0 cm diameter) in six-well plates at 1 × 10$^4$ cells per well and statically incubated for 28 days. The medium was changed every 3 days. On day 7, cell attachment and the coverage of the surfaces of the scaffolds were observed with SEM. On day 28, the samples were collected and prepared on 5 mm slides for 4',6-diamidino-2-phenylindole (DAPI) staining.

A part (80 mg) of each scaffold was added to 50 mL of PBS containing 0.02% bacteriostatic sodium azide and 0.2 mg/mL papain (Sigma-Aldrich) and incubated in a concussion incubator (25 rpm, 15°) at 37 °C for 4 weeks. Samples were retrieved every 7 days, rinsed with deionized water, and lyophilized. The change of mass in percentage loss was analyzed by comparing the mass with that of the samples before immersion. The enzymatic solution was changed every 7 days within the degradation.

5.5. Operative Procedure and Postoperative Care. This study was performed in strict accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, rev. 1985). Twenty male Sprague–Dawley rats, weighing 250–300 g, were obtained from SLAC National Rodent Laboratory Animal Resources (Shanghai, China). The Institutional Review Committee of Shanghai Tongji University School of Medicine approved all of the animal study protocols. The meshes were implanted as follows: under anesthesia induced with an intraperitoneal injection of 10% chloral hydrate solution, a midline skin incision was made and the subcutaneous tissue was dissected. A 30 × 20 cm$^2$ full defect was created. The rats were randomly assigned to the HAM–SF or SIS group ($n = 10$). An HAM–SF or SIS mesh, measuring 40 × 30 mm$^2$, was implanted into each rat, with the stromal side facing the peritoneal cavity, overlapping the edges of the defect by 5 mm. During surgery, the intestines were protected with moistened abdominal cloths. The mesh was fixed by suturing it intermittently with 3-0 polypropylene (Ethicon), and the skin was closed with 5-0 Vicryl interrupted sutures (Ethicon). Antibiotic prophylaxis [20 mg/(kg day) cefalexinum monohydrate] was diluted in water and administered orally for 3 days after surgery. After 28 days, the rats were sacrificed randomly with an intravenous injection of thiopental.

5.6. Macroscopic Observation. Signs of infection, dehiscence, hematoma, seroma, bulge, or herniation were recorded throughout the 28 day experiments. Visceral adhesion, including its tenacity and area, was scored by two investigators blinded to the group assignment, according to the following criteria: 0, no adhesion; 1, surface area <25%, freed with blunt dissection; 2, surface area <50%, freed with aggressive dissection; 3, surface area <75%, freed with sharp dissection; and 4, surface area ≥75%. The explants, including the muscular abdominal wall, were then retrieved from the
repair site for observation of the parietal side. The thickness of 10 random sites over the central area of the explant was measured to calculate the proportional change in thickness, and then the explants were collected for further examination.

5.7. Histological Analysis. The collected explants were embedded in paraffin, cut into 3.5 μm sections, mounted on slides, and stained with H&E to observe the general host response on the parietal side of the mesh. Masson’s trichrome staining was performed to detect the incorporation of the matrices, in terms of the organization and deposition of collagen. The samples were immunostained for collagen I (1:100; Sigma) and fibronectin (1:100; Sigma), which mainly indicate the development of adhesion, and visualized at the interface of mesh and peritoneum. The host inflammatory and immune responses, represented by leukocyte and macrophage infiltration, were evaluated by immunohistochemical staining with anti-CD54 (diluted 1:200; Santa Cruz Biotechnology) and anti-CD68 antibodies (diluted 1:200; Santa Cruz Biotechnology), respectively. Anti-CD31 immunofluorescent staining (1:100; Sigma) was used to assess explant vascularization. Positively stained vessels were quantified by counting them in 10 microscopic fields per sample. The results are expressed as the mean percentage of positively stained vessels (%) in the total number of sections for each explant. The data were analyzed by two blinded investigators with ImageJ software (NIH, Bethesda, MD).

5.8. Regulatory Factor Expression. To verify the expression of regulatory factor, including with anti-TGF-β1 antibody (1:50; Santa Cruz Biotechnology) and anti-HGF antibody (1:200; Sigma), immunofluorescent staining was visualized in the explants. To further quantify the specific regulatory factors related to the development of intraperitoneal adhesion, the proteins were extracted from the homogenates and western blotting was performed to determine their expression levels and determine potential interactions. The proteins were probed with antibodies directed against TGF-β1 (1:200; Santa Cruz Biotechnology), HGF (1:500; Sigma), and GAPDH (1:1000; Sigma) and detected as described above. Peritoneal fluid was collected from each rat to assess the extent of the intra-abdominal inflammatory response by measuring the levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) with ELISA kits (R&D Systems).

5.9. Mechanical Properties. The in vitro implants and in vivo explants were cut into 50 × 10 mm² strips (n = 5) for mechanical testing using a previously described method.17 The following mechanical properties were determined at room temperature after immersing the samples in PBS for 2 h to ensure their wettability before the tests: maximum load, Young’s modulus, tensile strength, and stiffness. In brief, the length of the sample held between two grips was set at 20 mm. The samples were stretched along their longitudinal axes at a speed of 10 mm/min until failure, and the maximum load in Newtons (N) was recorded with a uniaxial tensile testing machine (model S542, Instron, Norwood, MA). Young’s modulus (MPa) was calculated from the linear slope of the stress–strain curve, and the tensile strength was calculated as the maximal load divided by the cross-sectional area (MPa). Stiffness (N/mm) was determined by calculating the slope of the load (linear portion) vs displacement plot.

5.10. Statistical Analysis. Continuous variables are presented as mean ± standard deviation and analyzed with two-tailed Student’s t test or one-way analysis of variance followed by the Student–Newman–Keuls post hoc test. Ranked data were analyzed with the Kruskal–Wallis test. SPSS version 22.0 software (SPSS, Inc., Chicago, IL) was used for all statistical analyses. P < 0.05 was considered statistically significant.

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Z.L.: experimental design, photo taken, animal surgery, data collection, data analysis and interpretation, and manuscript writing; X.Z. and T.Z.: animal surgery, tissue histological collection, data analysis and interpretation, and manuscript writing; X.Z.: animal surgery, data collection, data analysis and interpretation, and manuscript writing. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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