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One and four layer acellular bladder matrix for fascial tissue reconstruction

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Abstract To determine whether the use of multiple layers of acellular bladder matrix (ABM) is more suitable for the treatment of abdominal wall hernia than a single layered ABM. The feasibility, biocompatibility and mechanical properties of both materials were assessed and compared. Biocompatibility testing was performed on 4 and 1 layered ABM. The matrices were used to repair an abdominal hernia model in 24 rabbits. The animals were followed for up to 3 months. Immediately after euthanasia, the implant site was inspected and samples were retrieved for histology, scanning electron microscopy and biomechanical studies. Both acellular biomaterials demonstrated excellent biocompatibility. At the time of retrieval, there was no evidence of infection. The matrices demonstrated biomechanical properties comparable to native tissue. Three hernias (25%) were found in the single layer ABM group and only 1 hernia (8%) was found in the 4 layer ABM group. Histologically, the matrix structure was intact and the cell density within the matrices decreased with time. The dominant cell type present within the matrices shifted from lymphocytes to fibroblasts over time. Both ABMs maintained adequate strength over time when used for hernia repair, and there was an extremely low incidence of adhesion formation. The single layer ABM showed enhanced cellular integration, while the 4 layer ABM reduced hernia formation. Either of these matrices may be useful as an off-the-shelf biomaterial for patients requiring fascial repair.

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1 Introduction

A variety of materials have been used as biomaterials for fascial repair, including synthetic implants and naturally derived tissue matrices [1–5]. An ideal biomaterial for fascial tissue repair should be biocompatible, promote new tissue formation, elicit minimal inflammation and be able to withstand sufficient tensile forces in order to maintain structural integrity in vivo [2, 3, 6]. Although synthetic materials, such as polypropylene mesh, have been used to match the required tensile strength for fascial tissue substitute, these materials are frequently associated with untoward effects such as fibrotic encapsulation, infection, erosion, bowel adhesion and mesh extrusion [4, 7–14]. Further, the resulting fibrous layer does not resemble normal facial tissue. As such, naturally derived processed tissue matrices, such as bovine pericardium or other acellular matrices, have been investigated due to their excellent biocompatibility [6, 15, 16].

Acellular matrices offer promising alternatives to synthetic polymeric materials for tissue repair and regeneration. These biomaterials can be processed in such a way as to retain growth factors, such as basic fibroblast growth factor (FGF-2) and transforming growth factor-beta (TGF-β) [17, 18] as well as glycosaminoglycans such as heparin and dermatan sulphate [19, 20]. They provide adequate microenvironment for cells that allows attachment, migration, proliferation and differentiation [21–23].

These naturally occurring biomaterials have been shown to integrate seamlessly with the host's tissues, induce the deposition of cells and additional extra cellular matrix (ECM), and promote rapid angiogenesis.

Acellular bladder matrix (ABM) is a collagen-based biomaterial processed from the bladder submucosa, known as the lamina propria. The matrix has been shown to be biocompatible [24–26], is processed easily, and has good tissue-handling characteristics. When ABM is used as a biological matrix, it permits early remodeling and vascularization, which can potentially increase resistance to infection and adhesion formation [22, 27–29]. This matrix has already been used experimentally and clinically for several applications [30–33]. Recently, ABM has been successfully used to treat urethral strictures in 9 patients [31]. However, ABM is relatively thin and may not possess the biomechanical strength and structure for applications that require high levels of resistive forces, such as the treatment of larger abdominal hernias. In such a case, it is uncertain whether multiple layers of ABM are needed to maintain the necessary strengths. In this study we manufactured a 4 layered ABM which resembles the abdominal apponeurosis (anterior abdominal wall) in structure, thickness and mechanical strengths to determine how this design would compare with the conventional 1 layer ABM as facial substitute in a rabbit model of abdominal wall hernia.

2 Materials and methods

2.1 Preparation of the acellular bladder matrix

Porcine bladders, obtained from IBP Inc. (Logansport, IN), were processed at ACell, Inc. (West Lafayette, IN). Briefly, each bladder was rinsed with running tap water, and placed in a −20°C freezer overnight. The bladder was opened and the muscle layer was excised with sharp scissors. The remaining tissue, consisting of the lamina propria (bladder submucosa), was placed in 0.9% normal saline. The tissue was disinfected in a solution containing peracetic acid, alcohol, and water for 2 h. This was followed by repeated rinse cycles using sterile phosphate buffered saline and water. Cells within the ABM were removed using the cell lysis technique described previously [34]. Briefly, bladder lamina propria was treated with distilled water in a stirring flask at 4°C for 1 day to lyse the cells in the tissue. Subsequently, the tissues were treated with 1% Triton X100 and 0.1% ammonium hydroxide in a stirring flask at 4°C for 14 days. The remaining collagen matrix was then rinsed with distilled water at 4°C for 2 days followed by phosphate buffered saline for 24 h.

The biomaterial was then cut into pieces using sterile instruments. Two layers of 4 × 4 sterile gauze were placed on a table in a 3 × 3 pattern (3 across and 3 down). A stainless steel mesh screen was placed on the gauze. Four layers of processed bladder tissue were then placed on the screen and air bubbles were removed from between the layers. A second stainless steel mesh screen, followed by a double layer of gauze, was placed on top of the bladder tissue. The apparatus was then sealed in nylon bagging film and connected to a vacuum for 4 h in order to compress and dehydrate the material. The layered bladder matrix was removed, frozen and lyophilized until completely dried. The matrix was trimmed to size, packaged in a foil pouch, E-beam sterilized and stored until used.

2.2 Biocompatibility

To evaluate whether the additional processing of the 4 layer ABM had any effect on biocompatibility, we assessed both ABM types using previously described methods [25]. Briefly, we investigated the effect of the biomaterial on cells in direct contact by mitochondrial metabolic activity assay (MTT) evaluating the mitochondrial function and by Neutral Red assay assessing cell viability. Primary mouse dermal fibroblasts were used for the biocompatibility testing. ABM fragments (0.03 cm^2/well) were placed in the center of subconfluent fibroblast monolayers in 96-well plates, according to established protocols [26]. Cell monolayers that were not exposed to the biomaterial served as negative controls, and latex fragments served as positive controls. The material-cell contact was maintained for 3 days at 37°C and 5% CO_2 before beginning each assay.

The mitochondrial metabolic activity in the cells exposed to the biomaterial was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using the direct contact method [25]. The cell layers were rinsed with PBS, and 125 µl of MTT (Sigma, Saint Louis, MI, USA, 1 mg/ml MTT in PBS containing 1 g/l glucose) was added to each well. After a 3-h incubation period at 37°C, the MTT solution was removed, and the insoluble Formazan crystals were dissolved using 100 µl of dimethylsulfoxide (Sigma). The intensity of the blue color obtained is directly proportional to the metabolic activity of the cells and inversely proportional to the toxicity of the material. The absorbance was measured at 540 nm using a 96-well plate spectrophotometer (ELX 800, BioTek Inc., Winooski, VN, USA).

The cytotoxic and bioactive effects of the biomaterial in direct contact with the cells were tested with Neutral Red according to published protocols [26]. The cell layers were rinsed with PBS, and 100 µl of Neutral Red Solution (Sigma, 0.005% in culture medium) was added into each well. After a 3 h incubation at 37°C, the Neutral Red solution was removed, and dye extraction was performed by adding 100 µl of 1% acetic acid in 50% ethanol solution

into each well. After placing the plate on a platform shaker for 5 min, the absorbance was measured at 540 nm using a 96-well plate spectrophotometer. The intensity of the red color is directly proportional to the viability of the cells and inversely proportional to the toxicity of the material. In both assays, cells grown in culture dishes without treatment served as controls (100%).

2.3 Animal studies

Acellular bladder matrices (ABM) were tested in 24 New Zealand White rabbits (approximately 3.5 kg, Millbrook Farms, Concord, Massachusetts) using an abdominal hernia model. The animals were randomized into 2 groups. In Group I, a single layer ABM was used to repair the hernias and in group II, a 4 layer ABM was implanted. After replacement of the abdominal wall with the ABM the animals were followed for up to 3 months. Tissue samples were retrieved after 1, 2 and 3 months and these samples were analyzed histologically, ultrastructurally and biomechanically. Native abdominal tissue was used as a control.

2.4 Anesthesia and surgery

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, and were performed in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The animals were sedated with an intramuscular injection of 10 mg/kg ketamine and an intravenous injection of 0.5 mg/kg acepromazine. After induction with propofol (4–8 mg/kg, IV), the animals were intubated and anesthesia was maintained with 2–3% isoflurane. The animals were placed in the supine position. The incision site was shaved and disinfected with iodine solution.

A midline abdominal incision was made and the subcutaneous tissue layer on the left side of the abdomen was bluntly dissected. Care was taken not to harm any internal organs. A defect (4 × 5.5 cm) was created by excising a full thickness portion of the abdominal wall. Then, the ABM selected for use in the repair surgery was soaked in saline for 5 min and trimmed to fit the defect. Although the single layer ABM possessed epithelial and serosal sides, visible identification of each was not feasible during the implantation procedures. Therefore, no preferential sides were applied for the surgery. When using the single layer ABM, the border of the biomaterial was folded at the edge to achieve a sturdier suture line. The ABM was sutured to the abdominal wall in a running fashion using Prolene 3-0 (non absorbable) sutures (Fig. 2). The wound closure was performed with subcutaneous 3-0 Vicryl (absorbable) and 4-0 PDS (absorbable) sutures for the skin. At the end of the procedure the animals were extubated. The animals were transferred to the postoperative care unit after antibiotic cream and a restraining collar were applied to protect the operative wound. All animals were placed in a post-operative recovery cage with a supply of 100% oxygen and heat for at least 4 h until fully awake. An intramuscular dose of Buprenorphine 0.02 mg/kg was given routinely every 12 h for the first 72 h post-operatively, and as needed thereafter. Fecal and urinary output were monitored and recorded.

2.5 Abdominogram

Abdominograms were performed by injecting 20 ml of contrast media (Optiray 240, Malinckrodt Inc, St. Louis, MO) into the abdominal cavity at the time of sacrifice. After waiting for several minutes for the contrast media to collect at the site of the hernia repair, lateral radiography was performed (Trex-net HR, Littleton, MA).

2.6 Implant retrieval and gross examination

Immediately after euthanasia, the implant site was inspected. Parameters assessed during gross examination included the integrity, size and integration of the biomaterial into surrounding connective tissue as well as the presence of fibrosis, infection and/or inflammatory response. The dimensions of each biomaterial segment were measured and the average change in size was expressed as a percent. The size of the biomaterials prior to surgery was defined as 100%.

The abdominal biomaterials were harvested through a midline incision. To reduce the risk of perforation of the matrix, the abdomen was opened with an incision through the rectus muscle on the opposing side. Adhesions to the biomaterial were scored using the Modified Diamond Scale ($0 = 0\%$, $1 \leq 25\%$, $2 = 25$–50%, $3 > 50\%$). After assessing the levels of adherence, the biomaterial was freed from the surrounding tissues and the entire implant was removed en bloc and measured. The harvested biomaterial was divided into 4 equal pieces for histology, biomechanical studies and scanning electron microscopy. A 5 × 5 cm piece of the contralateral external abdominal muscle was harvested to be used as a normal control. A hernia was defined as macroscopic rupture of the biomaterial at the time of retrieval.

2.7 Histology

The retrieved tissue specimens were fixed in 10% neutral Formalin. All specimens were rinsed 3 times in PBS and placed in the tissue processor (Citadel 1001, Thermo-Shandon). Five micron thick sections were obtained (RM2145, Leica), and these were stained with hematoxylin and eosin (H&E), a staining method widely accepted for

assessment of local inflammatory response. For each group, evidence of inflammatory response and the quality of the collagen layer were noted. To determine the cellular density within each biomaterial, the number of nuclei from 8 randomly selected high power fields were counted at each time point for each group.

2.8 Scanning electron microscopy

For ultrastructural analysis, implants were obtained at each time point and fixed in a 2.5% glutaraldehyde solution containing 0.085 M cacodylate buffer for 1 h. All samples were dehydrated through a graded series of ethanol and stored at 4°C. After critical point drying and sputtering (Hommur V) using a gold platinum target, the samples were analyzed using a scanning electron microscope (SEM, Leo 1450 VP) using 1500× magnification. Structural changes in the extracellular matrix were noted.

2.9 Biomechanical studies

Rectangular tissue strips measuring 20 mm × 6 mm were cut from each sample allowing 2 measurements per specimen. Tensile tests (Instron model 5544, MA, USA) were performed by applying a preload of 0.2 N and then elongating the tissue strips longitudinally at a speed of 0.05 mm/s until failure. The grip-to-grip spacing was 1 cm. All specimens were tested at room temperature and kept moist. The maximum tensile stress and strength were assessed. The results at the 0, 1, 2 and 3 months time points were compared and analyzed ($n = 4$). The tendonous portion of the native external abdominal muscle served as a normal control.

2.10 Porosity

The porosity of the biomaterial was measured by the liquid displacement and flow method [35]. When testing with the liquid displacement method, a piece of biomaterial was immersed in a known volume of ethanol (V1). The sample was kept immersed for 5 min until air bubbles were no longer observed. The total volume of ethanol and the ethanol-impregnated biomaterial was then recorded as volume (V2). The difference in volume (V2 − V1) is the volume of the biomaterial. The ethanol-impregnated biomaterial was then removed from the cylinder and the residual ethanol volume was recorded as V3. Porosity was obtained with the formula $E = (V1 - V3)/(V2 - V3)$. We also used the flow through method [35], which defines porosity as the flow of water through a biomaterial per unit time and per unit surface area at defined pressure. For this test, 0.5 cm^2 of wet biomaterial was exposed to static water pressure on one side. Leakage flow was collected over 1 min in a graduated cylinder for quantification of volume. The flow-through porosity was defined as the amount of water flowing through one square centimeter of construct, measured in milliliters per minute, at a pressure of 120 mmHg. All specimens were tested at room temperature and kept moist.

2.11 Statistical analysis

All presented data is expressed as mean ± standard error of the mean (SEM). For statistical analysis, SPSS v11 (SPSS Inc) software was used. One way analysis of variance with post hoc analysis (Bonferroni) was used for all analyses. A P-value $P < 0.05$ was considered significant.

3 Results

3.1 Biocompatibility testing

Both single layer and 4 layer ABMs demonstrated excellent biocompatibility (Fig. 1). Cell viability assays using Neutral Red showed that cells cultured in the presence of both the single layer ABM (108.4%, $P = 0.038$) and the 4 layer ABM (104.7%) led to cell viabilities equal to that seen in the positive controls (100%). Cells in direct contact with latex had significantly lower viabilities (5.3%, $P < 0.001$ for all comparisons to latex). A direct mitochondrial metabolic activity (MTT) assay indicated that 91.7% of the cells on the single layer ABM, 82.9% of the cells on the 4 layer ABM ($P = 0.01$) and 6.1% of the cells on latex were viable ($P < 0.001$ for all comparisons to latex). Taken together, no statistical difference between the single layer ABM and the 4 layer construct was found. However, the additional processing of the 4 layer ABM did not significantly decrease the outcome of these assays.

3.2 Gross examination

Two of the original rabbits were excluded from the study. One rabbit developed a hair ball problem which led to loss of over 10% of its bodyweight. The second animal developed a large hernia on the second day after surgery due to technical problems during the procedure. Both animals were sacrificed as recommended by the veterinary staff. Two additional rabbits were used to replace these animals. Therefore, a total of 24 animals were used and divided into two groups of 12 rabbits each.

At each of the defined time points, the implants and the surrounding tissues were inspected grossly after euthanasia (Table 1). All acellular grafts showed evidence of early vascularization, including visible blood vessels on the peritoneal side of the biomaterial. There were no signs of

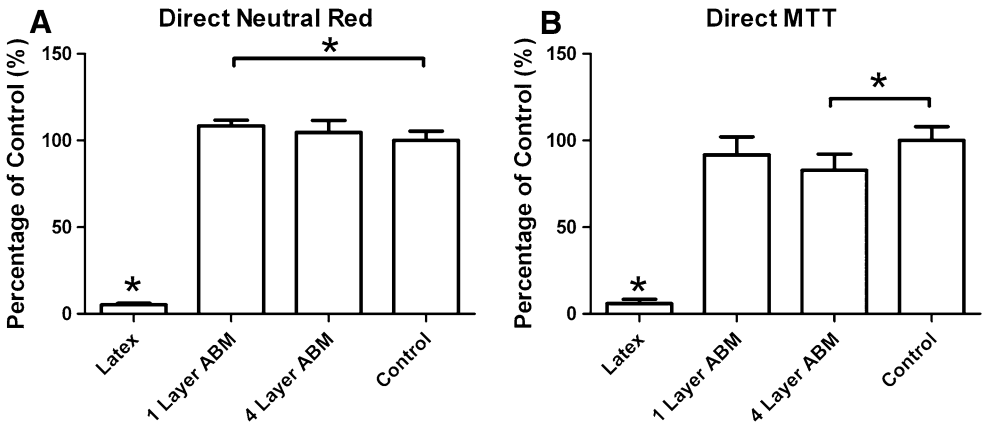

Fig. 1 Biocompatibility testing: **a** Cell viability assays using Neutral Red showed complete cell viability for both, the single layer ABM (108.4%) and the 4 layer ABM (104.7%), when compared to the positive controls (100%). Cells in direct contact with latex significantly decreased viability to 5.3% ($P < 0.001$ for all comparisons to latex). The single layer ABM performed significantly better then the control ($P = 0.038$), cells grown in culture dishes. **b** Direct mitochondrial metabolic activity (MTT) assay showed viability of 91.7% for the single layer ABM, 82.9% for the 4 layer ABM ($P = 0.01$) and 6.1% for latex ($P < 0.001$ for all comparisons to latex). Cells grown in culture dishes without treatment served as controls (100%)

Table 1 Macroscopic findings: a total of 4 animals experienced rupture of the biomaterial (hernia) at the time of retrieval

	Ruptures				Adhesions				Size		
	1 M	2 M	3 M	Total	1 M	2 M	3 M	Total	1 M	2 M	3 M
1 Layer ABM	2	1	0	3 (25%)	3 Grade I	2 Grade I	1 Grade I 1 Grade II	7 (58.3%)	105 ± 12%	94 ± 3%	105 ± 6%
4 Layer ABM	1	0	0	1 (8.3%)	2 Grade I	1 Grade I	3 Grade II	6 (50%)	105 ± 7%	96 ± 5%	85 ± 14%

All ruptures had developed during the early postoperative period. There was no evidence of infection or gross fibrosis in any of the specimens. In the single layer group only minimal adhesion was noted (grade 1), which consistently included the suture line. In the 4 layer group, 3 of 4 animals showed grade 2 adhesions at 3 months post-implantation. While the single layer ABM maintained the size after 3 months, the 4 layer ABM appeared to have contracted

inflammation, infection or fibrosis and the biomaterials were stably embedded into the host subcutaneous tissues. Further, no seromas of the biomaterials were recorded. Four animals developed herniation, which was defined as fascial rupture at the time of retrieval. All of these ruptures developed during the early postoperative period. In the single layer biomaterial group, the hernias occurred after 1 month ($n = 2$) and 2 months ($n = 1$). Only one hernia occurred in the 4 layer group, and it was found 1 month after surgery. While the intact 1 layer ABMs did not change in size after 3 months (105 ± 6%), the 4 layer ABM appeared to have contracted (85 ± 14%) ($P = 0.45$). Further, in 2 animals receiving 4 layer biomaterials, a thickened and retracted biomaterial was clearly visible and palpable at 3 months. The thickening was associated with the shrinkage of the ABM layer.

In general, the biomaterial integrated into the surrounding tissues in all of the samples (Fig. 2). There was no evidence of infection or gross fibrosis in any of the specimens. In the single layer group, only minimal adhesion was noted (grade 1), which consistently included the suture line. In the 4 layer group, 3 of the 4 animals showed a grade 2 adhesion with complete adhesion of the bladder after 3 months.

Abdominograms were performed after sacrifice in 3 animals per group. The radiographs showed a smooth contour of the external peritoneal layer. No contrast media appeared to leak into the abdominal tissue.

3.3 Porosity

The liquid displacement method used before implantation showed a porosity of 57.1 ± 4.6% for the single layer ABM and 50.5 ± 9.1% for the 4 layer ABM ($P = 0.4$). Before implantation and after harvest, samples of the single layer and 4 layer biomaterials from the animals were subjected to porosity measurements using the flow-through method. The single layer ABM demonstrated a porosity of 1.6 ml/min/cm^2 before implantation. In contrast, the 4 layer construct, as well as all harvested 1 and 4 layer

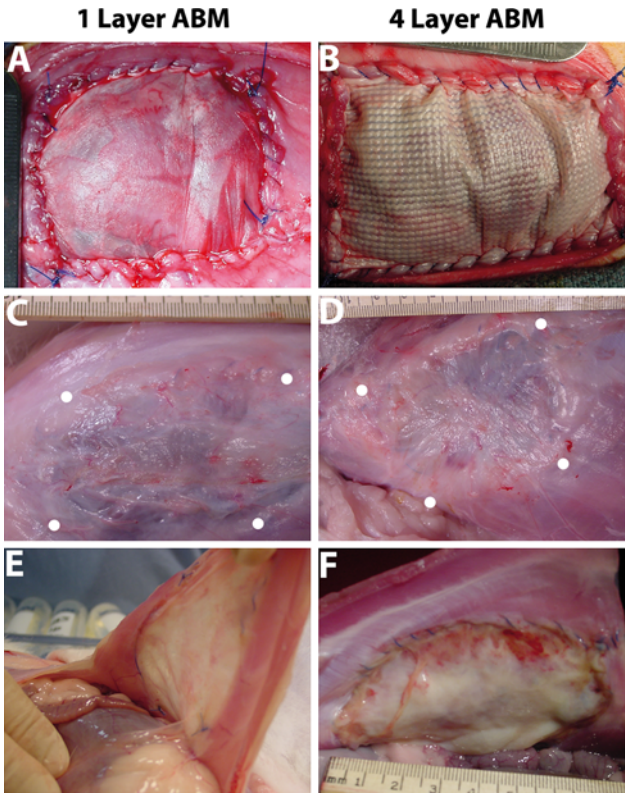

Fig. 2 Surgery and macroscopic findings: **a**, **b** Images showing the biomaterial in situ after implantation. **c**, **d** Macroscopic aspects at 3 months after implantation. The images demonstrate good integration of both biomaterials into the native fascia. *Dots* indicate the corners of the biomaterial. **e**, **f** Macroscopic view of the peritoneal side showing minimal adhesions and good integration into the abdominal wall (**a**, **c**, **d** single layer ABM; **b**, **d**, **f** 4 layer ABM)

samples, proved to be water tight. The SEM findings were consistent with these results.

3.4 Biomechanical studies

The biomechanical properties of the single and 4 layer ABMs were comparable to normal controls (Fig. 3). The single layer ABM had a maximum tensile stress of 3.1 ± 0.9 MPa prior to implantation, 2.4 ± 0.6 MPa at 1 month after implantation, 2.6 ± 0.3 MPa after 2 months and 3.7 ± 0.6 MPa after 3 months. The tensile stresses measured for the 4 layer ABM were 2.3 ± 0.3 MPa prior to implantation, 1.9 ± 0.4 MPa after 1 month, 3.6 ± 0.9 MPa after 2 months and 3.7 ± 1.1 MPa after 3 months. These results were comparable to the normal controls (3.6 ± 0.7 MPa). There was no significant difference in the maximum tensile stress (MPa) noted between the 0, 1, 2 and 3 month time points for both biomaterials ($P = 0.45$). However, a trend towards improvement in mechanical strength over time could be observed.

For potential materials to be used in abdominal hernia repairs, the strength (N/cm) is considered the most important factor [36]. Therefore, all measurements were expressed as strength (Fig. 3). The results of the strength testing indicate that there is no added benefit, in terms of mechanical properties, to using the 4 layer construct. The single layer matrices showed a tensile strength of 3.1 ± 1.2 N/cm prior to implantation, 7.9 ± 1.2 N/cm after 1 month, 13.5 ± 3.5 N/cm after 2 months and 10.5 ± 3.2 N/cm after 3 months. The findings for the

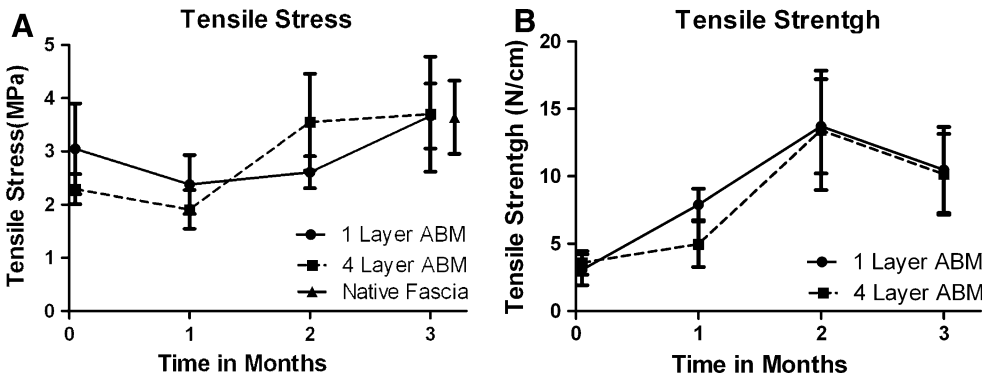

Fig. 3 Biomechanical testing: **a** Maximal tensile stress: the single layer ABM showed a maximum tensile stress of 3.1 ± 0.9 MPa prior to implantation, 2.4 ± 0.6 MPa after 1 month, 2.6 ± 0.3 MPa after 2 months and 3.7 ± 0.6 MPa after 3 months. The tensile stress for the 4 layer ABM were 2.3 ± 0.3 MPa prior to implantation, 1.9 ± 0.4 MPa after 1 month, 3.6 ± 0.9 MPa after 2 months and 3.7 ± 1.1 MPa after 3 months. These results were comparable to the normal controls (3.6 ± 0.7 MPa). There was no significant difference in the maximum tensile stress (MPa) noted, however a trend towards improvement of strength over time could be observed. **b** Maximal tensile strength (N/cm): the single layer ABM matrices showed a tensile strength of 3.1 ± 1.2 N/cm prior to implantation, 7.9 ± 1.2 N/cm after 1 month, 13.5 ± 3.5 N/cm after 2 months and 10.5 ± 3.2 N/cm after 3 months. The findings for the 4 layer matrices were 3.6 ± 0.9 N/cm before implantation, 4.9 ± 1.7 N/cm after 1 month, 13.4 ± 4.4 N/cm after 2 months and 10.2 ± 3.0 N/cm after 3 months. There was no significant difference in the maximum tensile strength (N/cm) noted between the 0, 1, 2 and 3 month time points for both biomaterials ($P = 0.10$)

4 layer matrices were 3.6 ± 0.9 N/cm before implantation, 4.9 ± 1.7 N/cm after 1 month, 13.4 ± 4.4 N/cm after 2 months and 10.2 ± 3.0 N/cm after 3 months. Native abdominal fascia has a strength of 19.6 ± 5.1 N/cm. There was no significant difference in the maximum tensile strength (N/cm) of the biomaterials ($P = 0.10$) at any of the observed time points.

3.5 Histology

Histological evaluation indicated that the matrix structure in both types of biomaterials was intact at all 3 time points. The amount of extra cellular matrix in the materials did not change over the study period. In implants harvested after 1 month, both materials contained a mixed cell infiltration composed of lymphocytes, plasma cells, and polymorphonuclear cells. In addition, foreign body granulomatous reactions with giant cells were present (Fig. 4). Giant cells were found at all time points, and the 4 layer implants contained larger numbers of these cells. No dystrophic calcification was present in any of the samples. Limited inflammatory infiltration was observed in the single layer constructs at 1 month post-implantation and at all time points in the 4 layer constructs. In the 4 layer constructs, the largest number of lymphocytes was observed at 3 months post-implantation, especially at the junction of the biomaterial and native fascial tissue. Angiogenesis with vascular infiltration was observed throughout the implanted matrices at all time points. The cell density within the implants decreased over time and a shift from dominance of lymphocytes to dominance of fibroblasts was observed. The cell density in the single layer ABM decreased from 195.0 ± 52.5 at 1 month to 147.8 ± 22.4 at 2 months to 96.3 ± 18.5 at 3 months. In the 4 layer ABM group, the cellularity decreased from 168.3 ± 6.7 at 1 month to 117.3 ± 5.7 at 2 months to 102.5 ± 10.9 at 3 months. There was no statistical difference between the groups at different time points ($P = 0.75$).

3.6 Ultrastructural analysis

Scanning electron microscopy (SEM) was used to evaluate the integrity of the ABM at each time point. The SEM images showed no differences in collagen structure at all time points (Fig. 5). The 4 individual layers of the 4 layer ABM were not distinguishable. Further, SEM imaging was able to underline the excellent biocompatibility of these materials by revealing a confluent peritoneal cell layer attached to the ABM at all time points (Fig. 6).

4 Discussion

Although many types of biomaterials have been used as fascial substitutes, none of these materials has been entirely satisfactory. Although propylene mesh has been used for abdominal hernia repair, untoward effects such as formation of a fibrotic capsule and discomfort due to the

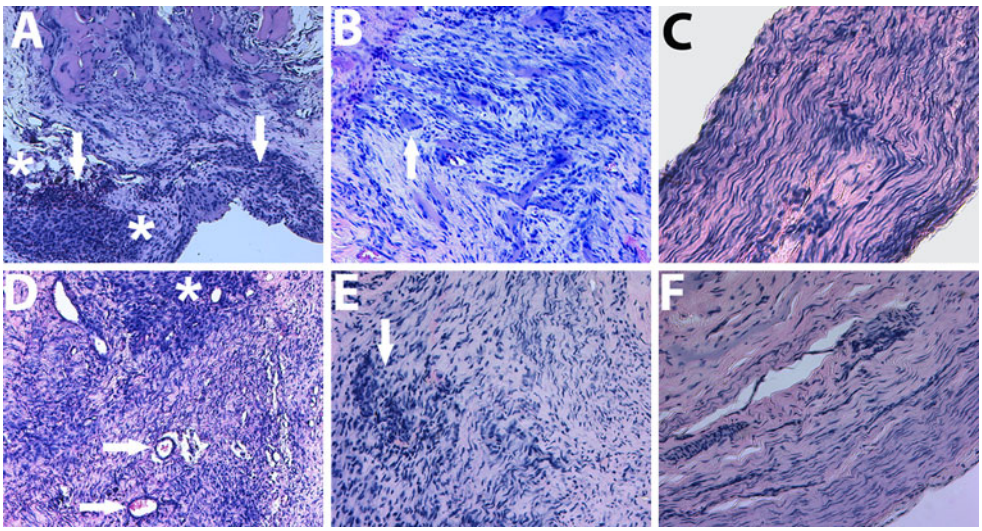

Fig. 4 Histologic analysis. Hematoxylin and eosin stained sections of both biomaterials (**a**–**c** single layer; **d**–**f** 4 layer ABM) showing early inflammatory response at 1 month with an inflammatory infiltration (**a**, *arrows*) and giant cells (**b**, *arrow*). This inflammatory response was most prominent at the interface between abdominal muscle and biomaterial (**a**, *asterisk*). In the single layer group the inflammatory cells were replaced with spindle shaped cells by 3 months (**c**). Both materials showed early vascularization, shown at 1 month (**d**, *arrows*) and inflammatory cells (*asterisk*). At 3 months the 4 layer ABM showed a reduction of inflammatory cells, however there were still more inflammatory infiltration present (**e**, *arrow*), when compared to the single layer biomaterial. The central regions of the 4 layer ABM demonstrated the formation of aligned spindle shaped cells by 3 months. All images reduced from 100×

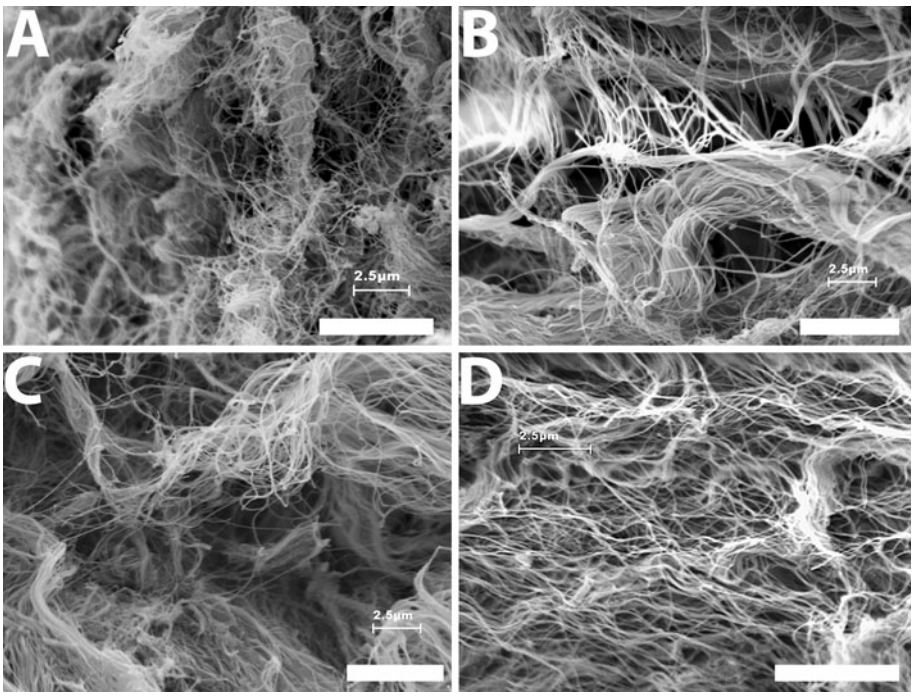

Fig. 5 Scanning electron microscopy (SEM): SEM showed the maintenance of the ultrastructural architecture, consisting of organized collagen bundles, at all time points. There was no significant degradation of the biomaterial within the study period. **a** Single layer ABM at 1 month, **b** single layer ABM at 3 months, **c** 4 layer ABM at 1 month, **d** 4 layer ABM at 3 months. Scale bar = 5 µm

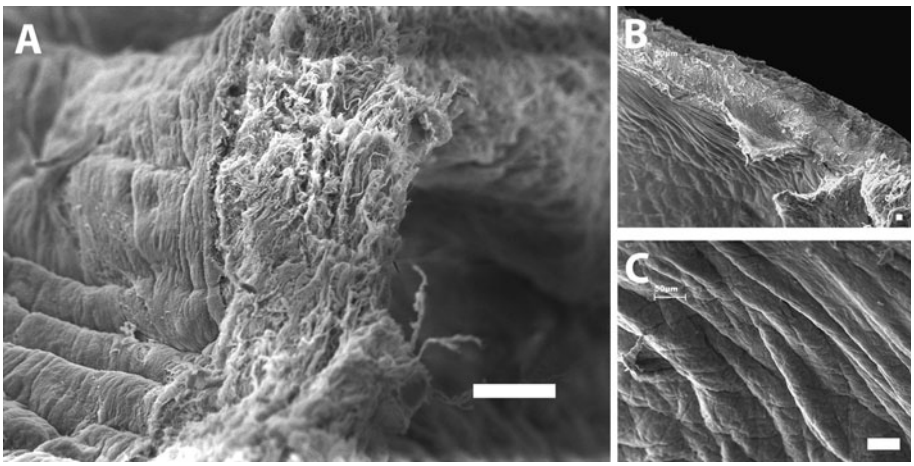

Fig. 6 Scanning electron microscopy (SEM): low power images of the biomaterial at 1 month show the excellent in vivo biocompatibility of this collagen based biomaterial. A confluent cell layer covering the peritoneal side of the biomaterial is evident. **a**, **b** Single layer ABM at 1 month. **c** 4 layer ABM at 1 month. Scale bar = 50 µm

resulting stiffness have remained as a problem [37]. Consequently, acellular biomaterials derived from tissues have been suggested as an alternative to polypropylene mesh in abdominal fascial repair surgery [6, 15, 16]. In this study, we tested a collagen-based matrix processed from porcine bladder in a rabbit abdominal hernia model. We show that both the single layer and 4 layer ABMs are biocompatible in vitro and in vivo, and that they are durable and effective when used as a fascial substitute.

ABM is processed by using a multistep detergent wash which removes nucleated cells and immunogenic proteins with minimal changes to the ECM and its mechanical properties. While collagen types I and IV are well preserved in the ABM, collagen type III remains within the biomaterial but is denatured [38]. Other ECM molecules including elastin, laminin and fibronectin are only slightly reduced in ABM. Interestingly, this biomaterial contains multiple growth factors which might support rapid cellular ingrowth and expansion [17, 18, 20].

One of the single most important factors considered in the selection of biomaterials for clinical applications is biocompatibility. Acellular matrix derived from bladder tissue is known to be biocompatible, and has been used experimentally and clinically in several applications [30–33]. Since the 4 layer ABM was processed in an industrial facility using established protocols, it was necessary to confirm the in vivo biocompatibility. Our results demonstrate excellent biocompatibility, both in vitro and in

vivo. None of the animals showed erosion, infection or graft rejection and all of the implanted ABM were securely embedded within the surrounding native fascia.

One of the cornerstones of a successful abdominal hernia repair is the selection of an appropriate biomaterial that can withstand changing forces, such as those that may occur during sneezing or coughing. By bonding multiple layers, ABM can easily be tailored to the thickness and size needed. We compared ABM biomaterials using biomechanical assessment as well as macroscopic and histologic findings. We examined the biomechanical properties of the implanted ABM grafts at each time point and showed that the tensile strength remained within normal limits during the entire study period. Moreover, the strength of both ABMs tended to increase over time. This may be due to the infiltration of fibroblastic host cells that produce new ECM. After 2 months, both materials displayed increased strength (N/cm) and were within the physiologic limits required for successful abdominal wall hernia treatment [36].

However, in our hands, the 4 layer ABM did not result in any significant improvement in biomechanical characteristics when compared to the single layer ABM. This might be due to the natural origin of the layers, which show a high variability within each layer. Further, manufacturing parameters such as dehydration time and technique, as well as sterilization method, are important parameters that could potentially affect the mechanical properties of ECM devices and should be taken into consideration when engineering tissue constructs [39–41]. On the other hand, the 4 layer matrix exhibited a water tight barrier in the flow-through porosity assessment. This type of material may be useful for selected applications where absolute water tightness is needed immediately upon implantation.

In this study we evaluated the biomaterials in a large abdominal hernia model. The removal of the abdominal wall in combination with the horizontal orientation of the rabbit body axis challenged the implanted biomaterials. 25% of the animals in the single layer group and 8% of the animals in the 4 layer group presented with a hernia after implantation. In all cases, the rim of the biomaterial remained on the suture line, indicating that the disruption had occurred through the matrix rather than at the suture line. The defects created by the suture needles may be an initiating factor for the rupture. Ko et al. have investigated acelluar matrix in a pig hernia model without removal of the abdominal wall. The authors conclude that a one layer acellular graft implanted using onlay, inlay or underlay techniques is sufficient for repair of ventral abdominal wall defects [42]. The use of a single layer ABM for full wall reconstruction, however, seemed insufficient and a multilayer ABM was thought to be favorable. Interestingly, the single layer ABM showed excellent stability in size with no contracture while the 4 layer ABM showed a significant shrinkage to 85% of the original size after 3 months. Two animals in the 4 layer ABM group showed a significant thickening of the biomaterial associated with severe shrinkage. Recent reports of complications for fascial replacement with 4 layer small intestine submucosa (SIS, Cook Biotech) demonstrated similar problems [43, 44]. The authors observed that the multilayer SIS led to increased fibrosis, possibly resulting from foreign body reactions and giant cell granulomatous inflammation. Even areas of chronic inflammation and focal areas of calcification have been reported in multilayered acellular grafts [44]. It is believed that the thickness of the collagen graft leads to a slower cellular remodeling, and that this contributes to the inflammatory reaction.

Histologically, both ABM grafts successfully integrated into native tissue. Infiltration of host inflammatory cells was observed during the early phase of implantation. However, the number of inflammatory cells present in the biomaterial decreased over time. The 4 layer ABM appears to extend the period of inflammatory infiltration delaying the ingrowth of fibroblasts. The single layer matrix showed an enhanced integration with the surrounding tissues. Substantial neovascularization was observed in all of the implants, indicating that the long-term survival of implanted grafts is likely.

Despite their differences, all ABM implants showed regional tissue formation with fibroblastic infiltration. These infiltrating fibroblasts demonstrated a longitudinal directional alignment in parallel to the abdominal wall. Further, none of the biomaterials induced severe bowel adhesions, which are commonly seen when synthetic biomaterials are used [45–48]. The adhesions seen in this study were limited mainly to the suture material used (Prolene, non absorbable) [49].

In conclusion, ABM is an off-the-shelf biomaterial that has demonstrated adequate in vivo biocompatibility. The ABM maintained its strength in the rabbit hernia repair model with an extremely low incidence of adhesion formation. The single layer ABM showed enhanced integration with the surrounding tissues, while the 4 layer ABM reduced hernia formation. However, the multilayer ABM appears to extend the period of lymphoid response, leading to follicle formation and delayed ingrowth of fibroblasts. Further studies are necessary to determine the clinical applicability of these materials.

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