Title: The Effect of Terminal Sterilization on the Material Properties and In-Vivo Remodeling of a Porcine Dermal Biologic Scaffold

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Abstract

Biologic scaffolds composed of extracellular matrix are commonly used in a variety of surgical procedures. The Food and Drug Administration typically regulates biologic scaffolds as medical devices, thus requiring terminal sterilization prior to clinical use. However, to date, no consensus exists for the most effective yet minimally destructive sterilization protocol for biologic scaffold materials. The objective of the present study was to characterize the effect of ethylene oxide, gamma irradiation and electron beam (e-beam) irradiation on the material properties and the elicited in vivo remodeling response of a porcine dermal biologic scaffold. Outcome measures included biochemical, structural, and mechanical properties as well as cytocompatibility in vitro. In vivo evaluation utilized a rodent model to examine the host response to the materials following 7, 14, and 35 days. The host response to each experimental group was determined by quantitative histologic methods and by immunolabeling for macrophage polarization (M1/M2). In vitro results show that increasing irradiation dosage resulted in a dose dependent decrease in mechanical properties compared to untreated controls. Ethylene oxide-treated porcine dermal ECM resulted in decreased DNA content, extractable total protein, and bFGF content compared to untreated controls. All ETO treated, gamma irradiated, and e-beam irradiated samples had similar cytocompatibility scores in-vitro. However, in-vivo results showed that increasing dosages of e-beam and gamma irradiation elicited an increased rate of degradation of the biologic scaffold material following 35 days.

1. Introduction

Biologic scaffolds composed of extracellular matrix (ECM) are commonly used in a variety of surgical applications to reinforce soft tissue, particularly in the abdominal wall and pelvic floor and in reconstructive breast surgery [1]. ECM scaffolds are produced by decellularization of source mammalian tissues and organs, including small intestine, urinary bladder, and dermis, among others. Over the last decade, a wide array of manufacturing protocols have been described for ECM scaffold materials, each of which vary widely in their use of chemical, enzymatic, and/or physical methods of decellularization. While it is inevitable that all processing methods used to prepare biologic scaffold materials will adversely affect the mechanical, biochemical, and cell signaling properties of the resulting ECM to some degree, the preferred methods will mitigate these effects as much as possible. Preservation of native ECM composition and ultrastructure in biologic scaffolds has been shown to facilitate beneficial constructive remodeling outcomes [2]. Specifically, the host response to biologic scaffold materials has been shown to be directly related to the efficacy of decellularization [3]. These findings have led to the proposal of a standard criteria for defining effective decellularization [2]. While much progress has been made to this end, relatively little has been studied regarding preferred methods of terminal sterilization.

The Food and Drug Administration (FDA) regulates biologic scaffolds derived from xenogeneic source tissue, including materials derived from decellularized porcine dermis, as medical devices; thus requiring these products to be terminally sterilized prior to clinical use. Common methods of terminal sterilization include electron beam (e-beam) and gamma (γ) irradiation and ethylene oxide [4] [4]. While it is known that each method exerts its sterilizing effect by modifying the structure or function of the critical components (e.g. proteins and nucleic acids) of target microorganisms and has directed guidelines regarding bacterial load (e.g., ISO/DIS 11135-1, ISO/DIS 11137-3), each method also has the potential to alter material properties of the ECM, including mechanics, susceptibility to degradation, biocompatibility, and ultimately the elicited *in vivo* remodeling response [5]. In contrast to the aforementioned decellularization criteria, currently no consensus exists for the most effective yet minimally destructive sterilization protocol for any biologic scaffold material. The objective of the present study was to characterize the effect of several types and doses of terminal sterilization on the material properties and elicited *in vivo* remodeling response of a biologic scaffold material derived from porcine dermis.

2. Methods

2.1. Experimental Design

The effect of terminal sterilization on a porcine dermal biologic scaffold was examined using *in vitro* and *in vivo* test systems. Porcine dermal ECM scaffold materials were exposed to one of three terminal sterilization methods – ETO, γ -irradiation, and e-beam irradiation. The γ -irradiation and e-beam irradiation groups were further subdivided into three dosage levels – 10, 25, and 40 kGy. All of the doses of sterilization evaluated within the current study are generally accepted to meet bioburden reduction criteria (i.e., a 6 log reduction). Quantification of bioburden reduction was not determined in the present study.”

Two non-sterilized control groups (non-sterilized porcine dermal ECM and non-sterilized intact porcine dermis) were also evaluated. All materials were evaluated for biochemical (DNA, sulfated glycosaminoglycan, & bFGF content), structural (scanning electron microscopy), and mechanical (thickness, porosity index, ball burst, suture retention) properties as well as cytocompatibility with human microvascular endothelial cells (HMEC-1). In the *in vivo* experiments, a rodent 1.5 cm x 1.5 cm bilateral partial thickness abdominal wall defect model was used to examine the host response to the materials following 7, 14, or 35 days (n=4 / group / time point). The host response to each experimental group was determined by quantitative histologic methods and by immunolabeling for macrophage polarization (M1/M2) within explanted specimens.

2.2. Preparation and Sterilization of Dermal ECM Scaffolds

Porcine full thickness skin from the dorsolateral flank of market weight pigs was harvested and processed immediately after euthanasia as previously described [6]. All full thickness skin sheets were cut into 35-cm x 50-cm rectangles. All samples were then mechanically delaminated to remove subcutaneous fat, excess connective tissue and the epidermis. While the mechanical delamination step is inherently variable, the allowed tolerances for these devices result in very small amounts of variability. Thus, the samples all started at the same thickness (i.e., they are not significantly different). The harvested sheets of porcine dermis were immediately frozen at -80 °C. Porcine dermis sheets designated to be treated with decellularization protocols were removed from the freezer and cut into sections measuring 3–7 cm x 3–7 cm. Dermis sections were decellularized as described previously [6]. Briefly, dermis was treated on a vortex shaker at 300 RPM at room temperature in the following solutions: 0.25% trypsin for 6 h, 1x; deionized water, 15 min, 3x; 70% ethanol, 10–12 h, 1x; 3% H₂O₂, 15 min, 1x, deionized water, 15 min, 2x; 1% Triton X-100 in 0.26% EDTA/0.69% Tris, 6 h, 1x and then overnight, 1x; deionized water, 15 min, 3x; 0.1% peracetic acid/4% ethanol, 2 h, 1x;

PBS, 15 min, 2x; and finally deionized water, 15 min, 2x. Following decellularization, all dermal ECM sheets were lyophilized. Lyophilized dermal sheets were sterilized with ETO gas (16 h cycle at 50 °C in a Series 3plus EO Gas Sterilizer, Anderson Sterilizers, Inc., Haw River, NC), γ -10 kGy, γ -25 kGy, γ -45 kGy, e-beam 10 kGy, e-beam 25 kGy, or e-beam 45 kGy.

2.3. Assessment of Cellular Content

Decellularization efficacy of dermis samples was assessed by three previously published criteria: (1) the absence of visible nuclear material on hematoxylin and eosin (H&E) stained and 40,6-diamidino-2-phenylindole (DAPI) stained sections; (2) a Quant-iT Pico-Green assay (Invitrogen, Carlsbad, CA) for quantification of double-stranded DNA; and (3) evaluation of a 2% agarose gel to determine the size of remaining DNA fragments [2].

2.3.1. Measuring DNA content

Scaffolds were digested in 0.6% proteinase K solution for at least 24 h at 50°C, until no visible tissue remained. Phenol/chloroform/isoamyl alcohol was added, and samples were centrifuged at 10,000g for 10 min at 4°C. The top aqueous phase containing the DNA was transferred into a new tube. Sodium acetate and ethanol was added to each sample, and the solution was mixed and placed at -80°C overnight. While still frozen, the samples were centrifuged at 4°C for 10 min at 10,000g. The supernatant was discarded, and all residual alcohol was removed. The pellet was suspended in TE (10 mM Tris / 1 mM EDTA) buffer. Double stranded DNA was quantified using Quant-iT PicoGreen Reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The dsDNA assay was performed twice (n=2) with two technical replicates per assay.

2.3.2. DNA Fragmentation Analysis

To determine the fragment size of remnant DNA, equal concentrations of extracted DNA from each sample were separated on a 2% agarose gel containing 0.5% ethidium bromide and visualized with ultraviolet transillumination using a reference 100-bp ladder (New England BioLabs, Ipswich, MA).

2.4. Sulfated Glycosaminoglycan Assay

Sulfated glycosaminoglycan concentrations of porcine dermis samples were determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd., Carrickfergus, Co Antrim, United Kingdom). Samples were prepared by digestion of 50 mg/ml dry weight of each sample with 0.1 mg/ml proteinase K in buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM

EDTA) for 48 h at 50 °C. Digested samples were assayed following the manufacturer's protocol, and the assay was performed in duplicate on three different samples.

2.5. Growth Factor Quantification

The ECM was lyophilized and milled using a 60-mesh screen (0.25 mm sieve size) to form an ECM powder. Three hundred (300) mg of ECM powder was suspended in 4.5 ml of urea–heparin extraction buffer. The extraction buffer consisted of 2 M urea and 5 mg/ml heparin in 50 mM Tris with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine and 10 mM N-ethylmaleimide) at pH 7.4. The extraction mixture was rocked at 4°C for 24 h and then centrifuged at 3000g for 30 min at 4°C. Supernatants were collected, and 4.5 ml of freshly prepared urea–heparin extraction buffer was added to each pellet. Pellets with extraction buffer were again rocked at 4°C for 24 h, centrifuged at 3000g for 30 min at 4°C, and supernatants were collected. Supernatants from first and second extractions were dialyzed against Barnstead filtered water (three changes, 80–100 vol. per change) in Slide-A-Lyzer Dialysis Cassettes, 3500 MWCO (Pierce, Rockford, IL). The concentration of total protein in each dialyzed extract was determined by the bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL), following the manufacturer's protocol, and extracts were frozen in aliquots until time of assay.

Concentrations of basic fibroblast growth factor (bFGF) in urea–heparin extracts of dermis samples were determined with the Quantikine Human FGF basic Immunoassay (R&D Systems, Minneapolis, MN). The bFGF protein was used as a general reference protein to assess the impact of the type and dose of terminal sterilization in this study on the amount of certain proteins with the scaffold material. Manufacturers' instructions were followed. Assays were performed in duplicate with three biologic replicates per sample (n = 3). The ELISA assay is cross-reactive with porcine growth factors and does not measure growth factor activity.

2.6. Scanning Electron Microscopy

Scanning electron micrographs were taken to examine ECM surface topology. Sterilized dermis samples were sputter coated with a 3.5 nm layer of gold/palladium alloy using a Sputter Coater 108 Auto (Cressington Scientific Instruments, Watford, UK) and imaged with a JEOL JSM6330f scanning electron microscope (JEOL, Peabody, MA).

2.7. Thickness Measurements of Sterilized Samples

Each experimental sample was measured for thickness by a blinded operator applying equivalent compressive force (8.5 ± 0.5 N) to all samples. Thickness after rehydration was measured by subjecting the specimens to lyophilization and then fully rehydrating the samples

by soaking in 0.9% sodium chloride solution for at least 48 h before measurement by the same operator in the same fashion. Fourteen samples were measured for each material.

2.8. Porosity Index

The hydrostatic porosity index (PI) measurement has been previously described [7]. Briefly, the test specimens were placed on the top orifice of the porosity testing apparatus. Distilled water was added to the upper reservoir and a hydrostatic pressure head of 120 mmHg was maintained throughout the duration of the test (3 min). To achieve this pressure head, a height of 163.2 cm was sustained between the water surface and the test specimen. A graduated cylinder was placed beneath the hydrostatic permeability testing apparatus and the clamp was released to allow passage of water to the test specimen. The clamp was closed after 3 min and the volume collected in the graduated cylinder was recorded. The test was performed with both sides of the material ($n = 6$ for each surface). The hydrostatic PI was calculated by dividing the volume collected by the area of the porosity testing apparatus' orifice and the duration of the test and reported as mL/min/cm².

2.9. Ball-burst Testing

The ball burst strength measurement has been previously described [8]. Samples from each test group were cut to a size of at least 6 cm x 6 cm and frozen at -80°C until time for testing (not more than 72 h). The specimens were rehydrated in 0.9% sodium chloride solution for approximately 15 min. Each specimen was mounted into a ball-burst fixture within the grips to prevent slippage. The ball-burst test, a measure of strength in response to multi-axial loading was conducted in compliance with the Standard Test Method for Bursting Strength of Knitted Goods, Constant-Rate-of-Traverse (CRT) Ball-burst Test (ASTM D 3787-89). A uniaxial tensile testing machine (MTS Insight; 2 kN capacity, MTS Systems Corp., Eden Prairie, MN) was equipped with a ball-burst compression cage in which a 25.4-mm (1-inch) polished stainless steel hemisphere rod was pushed against the material at a rate of 25.4 mm/min until failure. A total of 8 specimens were tested within each group.

2.10. Suture Retention Strength

The suture retention strength (SRS) test was performed according to ANSI/AAMI VP20–1994 Guidelines for Cardiovascular Implants-Vascular Prostheses. The SRS of a material is defined as the force required to pull a suture through the full thickness of the material at a specified bite depth and a specified pull rate. A 2–0 Prolene suture with a SH taper needle was passed through a square piece of test material with a 2-mm bite depth. The specimen was

clamped at one end while the suture was tied with a square knot and the loop attached to the Instron machine (Model 3345 Single Column Materials Testing System) and pulled at a constant rate of 10 cm/min. At least two tests were performed at locations that were 1.5 cm apart on the same edge of the square piece of each test material and the maximum load was recorded. At least 12 tests were performed for each group.

2.11. *In-vitro Cytocompatibility*

Dermal ECM sheets from each group were cut to 2 cm diameter circles for use in cell growth studies. Human microvascular endothelial cells (HMEC-1; a gift from Francisco J Candal, Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB-131 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 2 mM L-glutamine (Sigma), 100 U/mL penicillin/ 100 µg/mL streptomycin (Sigma), and 1 µg/mL hydrocortisone (Sigma) as previously described [9]. Cells were grown at 37°C and 5% CO₂, and media was changed every other day. After allowing the cells to propagate to sufficient numbers, test articles were briefly hydrated with sterile saline in sterile 6-well plates prior to cell seeding. Approximately one million cells were plated per test article. Test articles were seeded in quadruplicate. Cells were placed inside of stainless steel cell culture rings (inner diameter 1.3 cm) and allowed to attach to the test articles for 24 hours. Cell culture rings were removed and cell culture media was changed at 24 hours and every other day throughout experiments.

After 7 days of growth, cell culture media was removed and cells on test articles were immediately fixed in 10% Neutral Buffered Formalin for at least 18 hours. Following fixation, test articles were paraffin embedded, sectioned (5 µm), and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) and imaged for analysis. Eight representative microscopic images (40x magnification) were captured for each test article. All images were scored by five blinded investigators using a previously described scoring system based upon cellular confluence (0 to 100%), infiltration (surface only to 100% infiltration), and phenotype (0 to 100% normal) [10]. The confluence score is defined as the percentage of the surface covered with cells. A score of 100 would indicate a fully coated surface with adjoining cells and no gaps. The infiltration score is defined as the percentage of the total depth, in which cells have migrated within the tissue. For example, if cells are found halfway into the tissue, this would correspond to an infiltration score of 50. The phenotype score is defined as the percentage of healthy appearing cells. A healthy cell is flat and fully adhered to surrounding tissue and other cells. An unhealthy is round and not adhered to the surrounding tissue or other cells.

2.12. *Surgical Procedure and Test Article Collection*

All procedures were performed in accordance with the National Institute of Health (NIH) guidelines for care and use of laboratory animals and with approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Sprague Dawley rats (female; 250–350 g) were anesthetized with 1.5–3% isoflurane and maintained at a surgical plane of anesthesia. The surgical site was shaved, disinfected with a betadine solution, and an incision was made into the ventrolateral abdominal wall. Bilateral partial thickness abdominal body wall defects were created by excision of a 1.5 cm × 1.5 cm piece of tissue comprising the internal and external oblique muscles but leaving the transversalis muscle intact as described previously [11] (Figure 1A). Size matched dermal ECM scaffolds were then sutured into the defect site using nonresorbable 4-0 proline sutures at each of the 4 corners of the device. The skin was closed using 3-0 resorbable vicryl sutures.

Animals were sacrificed at 7, 14 or 35 days post implantation, and test articles were excised. Following euthanasia, the skin was gently dissected, reflected, and the entire body wall that includes the test or control article was explanted *en bloc*. The sample was then cut in half and each half immersed in 10% Neutral Buffered Formalin (NBF) for histologic analysis.

2.13. *Histologic Analysis of Tissue Explants*

Explanted test articles were paraffin embedded, sectioned (5 µm), and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) or Masson's Trichrome and imaged microscopically for analysis. Eight representative microscopic images (40x magnification) were captured for each test article across two distinct interfaces – within the center of the scaffold material and at the abdominal wall / ECM interface (Figure 1B). Five blinded investigators scored all of the images using a previously described semi-quantitative scoring system for cellularity, neovascularization, multinucleate giant cell formation, and morphologically degraded material (Figure 1B) [12, 13]. The morphologic appearance of the dermal ECM material was readily distinguishable from newly deposited connective tissue accumulated as a consequence of host remodeling. A subjective estimate of the amount of remodeled material was made, thus providing a qualitative assessment of the amount of remaining scaffold material.

2.14. *Macrophage Immunolabeling*

Immunolabeling of macrophages was performed on tissue sections from day 14 explants. Paraffin embedded sections were deparaffinized with xylene and rehydrated through a graded

ethanol series. Heat-mediated antigen retrieval was performed with 0.01 M citrate buffer (pH = 6) at 95–100 °C for 25 min. The tissue sections were subjected to Tris-Buffered Saline Tween-20 (TBST) for 15 min, followed by incubation in blocking buffer (2% horse serum albumin/1% bovine serum albumin/0.05% Tween-20/0.05% Triton X-100) for 1 h. The primary antibodies, diluted in blocking buffer, were added to the slides for 16 h at 4 °C in a humidified chamber. The slides were then washed three times in PBS prior to the addition of the secondary antibody for 1 h in a humidified chamber at room temperature. DAPI was used as a nuclear counterstain. The primary antibodies used in this study were mouse anti-rat CD68 (1:150, AbD Serotec, Raleigh, NC), goat polyclonal CD206 (Santa Cruz Biotech, Santa Cruz, CA), and rabbit anti-rat CD86 (1:150, Abcam, Cambridge, MA). The secondary antibodies used were Alexa Fluor® donkey anti-mouse 594 (1:200, Invitrogen), Alexa Fluor® donkey anti-goat 488 (1:200, Invitrogen) and donkey anti-rabbit IgG-PerCP-Cy5.5 (1:300, Santa Cruz Biotech, Santa Cruz, CA). CD68 is a pan-macrophage marker. CD86 is an M1 marker. CD206 is an M2 marker. All primary antibodies were confirmed to cross-react with rat epitopes. The sections were imaged at random fields along the interface of the native tissue and ECM scaffold. Quantification of M1/M2 polarization was achieved using a custom image analysis algorithm developed using the cell profiler image analysis package [13]. This algorithm identified and quantified the number of CD68⁺CD86⁺ (M1 phenotype) and CD68⁺CD206⁺ (M2 phenotype) cells present within the tissue sections. Any cells that co-expressed CD86 and CD206 were not counted. These numbers were then expressed as a ratio of M2/M1.

2.15. Statistical Analysis

Data sets were analyzed with either a one-way or a two-way analysis of variance (ANOVA) using SigmaStat 12.2 (sigma Aldrich, St. Louis, MO). The Student Newman-Keuls post-hoc test was used to locate the differences between means when the observed F ratio was statistically significant ($p < 0.05$). Data are reported as mean and standard error.

3. Results

3.1. Cellular Content

No intact nuclei were visible by H&E (Figure 2A) or DAPI staining (Figure 2B) in the decellularized samples. There was some diffuse positive DAPI stain that is likely representative of disrupted nucleic acid remnants from incomplete decellularization. However, all dermal samples contained less than 100 ng/mg dsDNA following decellularization (Figure 2C). The

ETO sterilized samples contained 30 ± 10 ng/mg which was significantly lower ($p < 0.01$) than all other sterilized samples. All DNA remnants were present in fragments less than 200 bp in length (Figure 2D).

3.2. Biochemical Properties

The amount of urea-heparin extractable protein varied across sterilization treatments (Supplemental Figure 1). Extracted protein per gram of dry weight increased with increasing doses of γ and e-beam irradiation. ETO treated dermal ECM had the least ($p < 0.05$) amount of extractable protein. The amount of bFGF in the ECM samples (Figure 3) was similar across groups with the exception of ETO, which had less ($p < 0.01$) extractable bFGF than scaffolds from the other sterilization conditions. The amount of sGAGs in the test samples was equivalent to non-sterilized control values (405 ± 12 $\mu\text{g/g}$; Data not shown).

3.3. Ultrastructural Properties

Scanning electron micrographs show that dermal ECM scaffolds from all experimental conditions show similar surface topology as control samples (Figure 4).

3.4. Mechanical Properties and Porosity

The thickness of the scaffold was not affected by type or level of sterilization (Supplemental Figure 2). Multiaxial strength of the test articles following ETO, γ and e-beam exposure was compared to controls (Figure 5). Burst strength of the ETO-treated sample was similar to that of the control, whereas there was a dose-dependent decrease ($p < 0.01$) in the burst strength of scaffolds exposed to increasing dosages of irradiation (Figure 5A). Suture retention strength was not significantly altered by exposure to ETO and 10 kGy of γ and e-beam irradiation (Figure 5B). However, higher doses of irradiation (i.e., 25 kGy and 40 kGy) resulted in a decreased ($p < 0.01$) suture retention strength compared to the control samples.

Porosity index levels trended towards a lower index level with decreasing doses of γ and e-beam irradiation, when compared to the non-sterilized control (Figure 5C). Gamma and e-beam at 10kGy had the lowest porosity index in their respective groups. The ETO treatment group was found to have the lowest porosity index level compared to control, γ and e-beam. However, meaningful differences between the non-sterile control and treatment groups were not observed in this study perhaps based on the inherent variability of biologic scaffolds or the n-value used in the present study.

3.5. Cytocompatibility

All dermal ECM test articles showed equivalent cytocompatibility scores for HMEC cells at the time point studied. After 7 days in culture, the HMEC cells reached approximately 60% confluence with minimal infiltration and essentially normal cell phenotype (Figure 6).

3.6. Host Response

The host response to dermal ECM scaffolds was evaluated at 7, 14, and 35 days (Figure 7). Gross images of the test article in-situ at time of sacrifice show that the test articles were clearly identifiable at all time points with the exception of those test articles subjected to 40 kGy doses of γ and e-beam which were nearly transparent at 35 d post-op (Figure 7A). These observations were corroborated by representative Masson's Trichome stained histologic sections where less scaffold (indicated by blue color) was present at the higher doses of irradiation compared to the ETO and 10 kGy irradiation samples (Figure 7B). Qualitatively, cell infiltration into all scaffolds increased with time. Little or no cell infiltration was present at 7 days and increasing depth and number of cells was present at 14 and 35 d post operatively, respectively (Figure 7C).

Histologic scoring showed the highest level of cellularity in 40 kGy γ sample at 14d and 40 kGy e-beam sample at 35d (Figure 8A). Vascularity in the samples was largely the same in all test articles with the exception of the 35d e-beam 40 kGy samples (Figure 8B). Similarly, the 40 kGy e-beam samples had the most multinucleate giant cells (MNGCs) among test articles, although it should be noted that on average there were still less than 1 MNGC per high-powered field of view (Figure 8C). Qualitative histologic scoring showed that the 40 kGy e-beam at 35d had the greatest degree of degradation (Figure 8D).

Macrophage immunolabeling at 14d post-operative showed a similar M2/M1 ratio among all test articles **with the exception of 25kGy γ which had an increased M2/M1 ratio** (Figure 9A). Total number of infiltrating macrophages was similar across groups with the exception of 40kGy γ , which had an increase in macrophage number at 14d post-surgery ($p < 0.01$, Figure 9B).

4. Discussion

The present study shows that increasing dosage of irradiation results **in increasing damage to the ECM scaffold as evident by an increased post-sterilization scaffold extractable protein** and a dose dependent decrease in mechanical properties compared to untreated controls. ETO-treated porcine dermis ECM resulted in decreased DNA content, total protein extracted, and bFGF content compared to untreated controls. All ETO, γ , and e-beam treated samples had similar cytocompatibility scores in-vitro. However, in-vivo results show that increasing amounts

of e-beam and γ irradiation elicited an increased rate of degradation of the biologic scaffold material following 14 and 35 days, respectively.

The FDA's classification of biologic scaffold materials as medical devices requires terminal sterilization to assure sterility guidelines are met. Common terminal sterilization methods, such as ETO exposure and irradiation, can achieve effective sterilization but these processes are known to affect the ultrastructure and mechanical properties of the ECM [14]. Material and structural properties, such as burst strength and stiffness, are important parameters for clinical applications such as ventral hernia repair [15]. Although ECM scaffolds tend to degrade quickly after implantation due to the host tissue response and the remodeling process, initial material and structural properties are important when the scaffold is intended for load bearing applications [16]. The present study shows that exposure to high levels of e-beam and γ irradiation results in a dose-dependent decrease in the multiaxial strength and suture retention strength of the material.

Previous studies have described effects of terminal sterilization upon the mechanical properties of tissue grafts and different naturally derived biomaterials [17]. However, these studies included materials which differed in preparation methods, hydration states during sterilization, and sterilizing dosage; therefore direct translation of this information to the sterilization method effects on biologic scaffolds is confounded. Since each method of sterilization has distinct effects on ECM constituents (e.g., collagens, proteoglycans, and glycosaminoglycans), a valid comparison of sterilization methods requires a direct comparison of the effects upon the same starting material and structural properties. The present study used the same starting substrate (i.e., decellularized porcine dermis) and therefore the reported differences in host response and material properties are directly attributable to the method and dose of sterilization.

ETO is a chemical sterilant with an unstable ring structure that alkylates functional groups (amino, carboxyl, hydroxyl, and sulfhydryl) resulting in the inactivation of nucleic acids and proteins [18]. Previous studies have shown little or no change in the mechanical properties of a device following ETO sterilization [17]. The present study showed that ETO exposure resulted in changes to the biochemical composition but, consistent with previous studies, the ultrastructure and mechanical properties were largely unchanged compared to untreated controls. ETO treatment can leave toxic residues that have been implicated as the cause of pro-inflammatory processes and poor outcomes [19]. The toxic residues dissipate quickly however and it is unlikely that notable amounts remain within a week of ETO exposure. Sterilization with ETO did not adversely affect in-vitro cellular growth and the in-vivo host response in the present study.

The most common technique for sterilization of biologic scaffolds is ionizing radiation, including e-beam and γ irradiation. Radiation exerts sterilization by the production of free radicals that are thought to result in scission of peptide backbones, disruption of hydrogen bonds, and crosslink formation [20]. Previous studies have shown a reduction in mechanical properties of biologic scaffolds following ionizing radiation sterilization [21]. Similarly, radiation has been shown to impair the attachment and growth of cells on biologic scaffolds [22]. Results of the present study showed irradiation results in alterations to the collagen fiber architecture and a dose-dependent decrease in both suture retention strength and maximum tangential stiffness. Radiation did not significantly change in-vitro cytocompatibility but at higher doses of radiation the scaffolds degraded more rapidly in-vivo than those exposed to low dose irradiation or ETO, likely due to increased scission of collagen backbone. The porcine dermal specimens exposed to high dose radiation were also associated with an increased infiltration of macrophages.

There are several key variables associated with the terminal sterilization of an ECM scaffold material. For example, hydration status (lyophilized vs hydrated) and temperature (room temp vs frozen) of the ECM scaffold during sterilization have been suggested to affect the material properties. Additionally, although the present study was conducted upon extracellular matrix derived from porcine dermis, it is unknown whether or not the results will be applicable to the preparation of biologic scaffolds from other tissues and organs. Studies should be conducted to systematically address these areas in an effort to further improve outcomes.

5. Conclusion

The present study shows that 40 kGy of e-beam and γ irradiation induce an adverse effect on the material properties and change the response to a porcine dermal biologic scaffold both *in vitro* and *in vivo*. However, effective methods of terminal sterilization can be used with minimal effects upon the structure and function of biologic scaffold materials. These findings highlight the importance of selecting an appropriate type and dose sterilization for biologic scaffold materials to optimize their material properties and performance *in vivo*.

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Figure Legends

Figure 1: Overview of animal model and histologic evaluation scheme. **(A)** Schematic of animal model and histologic cross section. The green “x” represents site of suture placement at corners of each device (yellow squares). The material is explanted en bloc and bisected. The histologic cross section depicts the rat abdominal wall defect, where the external and internal oblique is removed and replaced with a dermal ECM scaffold material. **(B)** Table describing the scoring system used to evaluate the histologic sections. **(C)** Schematic of histologic evaluation of explanted materials. Colored boxes represent the approximate location of 20X images used for histologic evaluation for each location.

Figure 2: Decellularization Quantification. Cellular content of dermis samples was assessed by previously published criteria: the absence of visible nuclear material on hematoxylin and eosin (H&E) stained **(A)** and 40,6-diamidino-2-phenylindole (DAPI) stained sections **(B)**; a Quant-iT Pico-Green assay for quantification of double-stranded DNA **(C)**; and evaluation of a 2% agarose gel to determine the size of remaining DNA fragments **(D)**. **n = 3** * $p < 0.05$, ** $p < 0.01$ as compared to control.

Figure 3: Biochemical Composition. Concentration of basic fibroblast growth factor (bFGF) in urea–heparin extracts of samples were determined with the Quantikine Human FGF basic Immunoassay **(A)**. All results were normalized to dry weight tissue. Assays were performed in duplicate on three independent samples for each treatment group. **N = 3** * $p < 0.05$, ** $p < 0.01$ as compared to control.

Figure 4: Scanning Electron Microscopy. SEM was used to examine the surface topology of each experimental group. SEM images were captured using a JEOL 6335F Field Emission SEM instrument with a backscatter detector. **Scale bar = 200 μm .**

Figure 5: Mechanical Properties & Hydrostatic Porosity Index. The ball-burst test conducted in compliance with the Standard Test Method for Bursting Strength of Knitted Goods, Constant-Rate-of-Traverse (CRT) Ball-burst Test (ASTM D 3787-89) (**A**). The suture retention test was performed according to ANSI/AAMI VP20–1994 Guidelines for Cardiovascular Implants-Vascular Prostheses (**B**). The hydrostatic PI was calculated by dividing the volume collected by the area of the porosity testing apparatus' orifice, and the duration of the test (**C**). $n = 6$ * $p < 0.05$, ** $p < 0.01$ as compared to control.

Figure 6: Cytocompatibility. HMEC were grown on each test article for 7 days and subsequently scored for cellular confluence, infiltration, and phenotype. $n = 4$ with 4 technical replicates. # $p < 0.05$, ## $p < 0.01$ as compared to control.

Figure 7: In Vivo Remodeling Response. Following euthanasia, the skin was gently dissected, reflected, and photographs were taken of each animal and each test or control article *in situ* (**A**). The entire body wall that includes the test or control article was explanted *en bloc*. The sample was then cut in half and submitted for histological analysis. Masson's Trichrome stained slides were mosaic imaged to illustrate the entire cross section of each test article at each time point (**B**). Additional slides were stained with H&E and imaged for histomorphologic analysis. Representative microscopic images (40x) were captured for each test article (**C**).

Figure 8: Semi-Quantitative Histomorphologic Scoring. Semi-quantitative scoring of each interface of each device was conducted for cellularity (**A**), neovascularization (**B**), multinucleate giant cell formation (**C**), and material degradation (**D**). Five blinded individuals scored each image independently and the average score was used to compile the results. $n = 4$ biologic replicates with 8 technical replicates * $p < 0.05$, ** $p < 0.01$ as compared to control.

Figure 9: Macrophage Phenotype Assessment. Quantification of M1/M2 polarization was achieved using a custom image analysis pipeline developed using the cell profiler image analysis package. This custom pipeline identified and quantified the number of CD68⁺CD86⁺ (M1 phenotype) and CD68⁺CD206⁺ (M2 phenotype) cells present within the tissue sections. These numbers were then expressed as a ratio of M2/M1. Any cells that co-express the CD86⁺ and CD206⁺ markers were counted separately. $n = 4$ biologic replicates with 8 technical replicates * $p < 0.05$, ** $p < 0.01$ as compared to control.

Supplemental Figure 1: Extractable Protein Content in Sterilized Samples. The soluble protein concentration in sterile samples was compared to the non-sterile control using a BCA assay. Amount of soluble protein varied dependent upon sterilization treatment and dose. * $p < 0.05$, *** $p < 0.01$

Supplemental Figure 2: Scaffold thickness. Independent of hydration state, the thickness of the dermal scaffold was not affected by type or level of sterilization.

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A

Partial Thickness Abdominal Wall Defect
(1.5 x1.5 cm) with material inlay

B

	Stain	Magnification	Location of measurement
Cellularity	H&E	40x	4 locations within ECM & ECM- host tissue interface
Neovascularization	H&E	40x	4 locations within ECM & ECM- host tissue interface
Multinucleate giant cell formation	H&E	40x	4 locations within ECM & ECM- host tissue interface
Morphologically “degraded” material (%)	H&E	40x	4 locations within ECM & ECM- host tissue interface

C

Within dECM
Abdominal Wall / dECM Interface

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9