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Effects of sterilization on an extracellular matrix scaffold: Part I. Composition and matrix architecture

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Abstract The impact of peracetic acid (PAA), lyophilization, and ethylene oxide (EO) sterilization on the composition and three dimensional matrix structure of small intestinal submucosa (SIS), a biologic scaffold used to stimulate the repair of damaged tissues and organs, was examined. Fibronectin and glycosaminoglycans are retained in SIS following oxidation by peracetic acid and alkylation using ethylene oxide gas. Significant amounts of FGF-2 are also retained, but VEGF is susceptible to the effects of PAA and is dramatically reduced following processing. Further, matrix oxidation, lyophilization, and sterilization with EO can be performed without irreversibly collapsing the three dimensional structure of the native SIS. These structural features and growth promoting extracellular matrix constituents are likely to be important variables underlying cellular attachment, infiltration and eventual incorporation of SIS into healing host tissues.

1 Introduction

Implantation of a graft material can improve the natural wound healing environment if the graft mimics the natural structure and composition of the surrounding implant site. While synthetic materials may be fabricated to mimic the three-dimensional architecture of the surrounding tissues, biologic scaffolds derived from extracellular matrix (ECM) can be implanted in their natural forms to achieve the same result. The challenge in preparing these biologic scaffold materials for clinical use, however, lies in retaining the natural structure and composition of the ECM while ensuring its safety for clinical implantation.

Processing methods used to achieve clinical safety often include harsh steps that subject the ECM to acids, enzymes, or other chemical treatments. These steps can denature the biomaterial, eliminate its inherent bioactivity, and prevent its ability to interact with the patient's cells. For example, crosslinking agents such as glutaraldehyde or hexamethylene diisocyanate (HMDI) are often used to increase implant strength and attempt to reduce their antigenicity. These compounds, however, also reduce the ability of cells to interact with the treated material [1], cause cutaneous sensitization [2], and lead to calcification [3]. Enzymes such as trypsin, amylase, and neuramidase are also often used to reduce rejection potential because they semi-selectively remove matrix components from the finished product. Such treatments, however, also remove potentially valuable matrix constituents, such as growth factors and glycosaminoglycans. Chemicals such as hydrogen peroxide or peracetic acid are often used as disinfectants, but these oxidize the biomaterial and may reduce the structural integrity of the collagen fibers. Oxidative modification of glycosaminoglycans fractures them and impairs their ability to interact with growth factors and other essential matrix components [4], while oxidation of proteins often renders them inactive, eliminating their bioactive properties.

Previous studies suggest that the composition and activity of the ECM and its constituents can be retained during processing if individual protein components are sequestered naturally [5] or in combination with other carriers [6–9]. Some growth factors, such as FGF-2, are inherently more stable than others and are even able to retain their activity

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under acidic and oxidizing conditions in the absence of stabilizing agents [10]. The retention of bioactivity of matrix proteins following lyophilization, a common protein stabilization method, has also been reported [11–13]. The osteoinductive properties of demineralized bone been shown to be retained following sterilization with ethylene oxide (EO) [14].

Small intestinal submucosa (SIS) derived from the porcine jejunum has been used as an acellular biologic scaffold in surgical applications [15–18]. It has also been used to stimulate the closure of chronic non-healing wounds [19]. In its natural form, SIS consists primarily of several types of collagens [20], with smaller, but significant, amounts of glycosaminoglycans [21], glycoproteins [22], and growth factors [23, 24]. However, its three-dimensional architecture and composition following processing to ensure clinical safety has not been previously examined. The purpose of this study, therefore, was to determine if the known glycosaminoglycan content, growth factor composition, and three dimensional architecture of SIS are retained following treatment with an oxidizing agent, following lyophilization, and after sterilization with EO gas.

2 Materials and methods

2.1 Reagents

Peracetic acid (PAA) was obtained from FMC (Chicago, IL). Human IgG agarose was purchased from Sigma (St. Louis, MO). ELISA detection kits for active TGFβ1, VEGF, and FGF-2 were from R&D Systems (Quantikine ELISA, Minneapolis, MN). The ELISA detection kit for fibronectin was from Chemicon (Temecula, CA). The Blyscan Assay was from Biodye Science (Newtownabbey, Northern Ireland). The hyaluronic acid (HA) detection kit was from Corgenix, Inc. (Denver, CO). All other chemicals and reagents were purchased from Sigma or as otherwise indicated.

2.2 Procurement of small intestine submucosa (SIS)

Sections of porcine jejunum were obtained immediately following slaughter. The intestine was rinsed in water to remove its contents and then split longitudinally to form a sheet. The tunica muscularis externa, the tunica serosa, and the superficial layers of the tunica mucosa were removed by mechanical delamination. The resultant SIS was then thoroughly rinsed in deionized water and stored at 4 °C prior to further evaluation.

2.3 PAA disinfection of SIS

Sections of porcine jejunum were subjected to treatment with a dilute solution of PAA for two hours at room temperature using methods described elsewhere [23, 25]. The tunica muscularis externa, the tunica serosa, and the superficial layers of the tunica mucosa were then removed by mechanical delamination. The PAA-treated SIS (SIS_{PAA}) was stored at 4°C in sterile containers prior to further evaluation.

2.4 Lyophilization & ethylene oxide sterilization

SIS_{PAA} was frozen and lyophilized to produce a dry sheet. Following lyophilization, the SIS_{PAA} was packaged into gas permeable pouches and sterilized with EO gas. EO-sterilized SIS (SIS_{EO}) was stored under sterile conditions at room temperature prior to evaluation.

2.5 TGFβ1, VEGF, and FGF-2 detection

SIS, SIS_{PAA} or SIS_{EO} were cut into 0.5-cm^2pieces using sterile instruments. The tissue was extracted at 4°C under constant stirring for 24 hours in either 4M guanidine hydrochloride (for TGFβ1 detection) or 2M urea (for FGF-2 and VEGF detection) containing 2.5 mg/ml heparin (10 g tissue/100 ml extraction buffer) and a protease inhibitor cocktail (10 mM *n*-ethylmaleimide, 1 mM PMSF, 5 mM benzamidine, 10 mM EDTA, 50 mM sodium acetate, 100 mM ε-amino-*n*-caproic acid). After 24 hours, the extracted tissue was transferred to centrifuge tubes and the insoluble fraction pelleted at 12,000 $\times g$ (30 min, 4°C). The supernatant was transferred to dialysis tubing (MWCO 3500) and dialyzed exhaustively against sterile water. Following dialysis, the dialyzed material was centrifuged at 12,000 $\times g$ to remove any additional particulate matter, and the supernatant was filtered through a 0.45 μm filter. The extract was lyophilized. A sample of the lyophilized extract was reconstituted in sterile water at 10 mg/ml and mixed with human IgG agarose for 60 min, centrifuged briefly to settle the beads, and stored at 4°C for evaluation by ELISA.

Extracted samples of SIS, SIS_{PAA} and SIS_{EO} were assayed for the presence of TGFβ1, VEGF, and FGF-2 according to the instructions provided by the assay kits. Each sample was run at three different dilutions in duplicate wells. The color reaction was measured at 450 nm when the maximal absorbance on the plate reached approximately 1.0. The data was plotted as background-corrected absorbance vs. concentration. The amount of TGFβ1, VEGF, and FGF-2 in the extracted samples was quantified and back-calculated to the amount of starting material in order to yield a measure of each growth factor based on the total original dry tissue weight.

2.6 Fibronectin detection

SIS, SIS_{PAA} and SIS_{EO} were prepared for the fibronectin ELISA by homogenization in 500 μl phosphate buffered saline (PBS). Briefly, 25 mm^2 samples of each ECM were placed in 1.5 ml centrifuge tubes containing 500 μl of chilled PBS. Samples were ground for 30 seconds using a tissue homogenizer, and allowed to sit on ice for 10 minutes. The grinding procedure was repeated three times, samples were centrifuged to pellet the insoluble fraction, and the supernatant was recovered.

The fibronectin ELISA was performed as directed. Briefly, 100 μl of sample and controls were incubated with 100 μl of rabbit anti-human fibronectin solution for 10 minutes at room temperature (RT) in a 96-well assay plate. One-half of the sample was transferred to the appropriate well of an ELISA assay plate and incubated for 1 hour at RT. After washing, 100 μl of HRP-linked goat anti-rabbit solution was added to each well and allowed to incubate for 30 minutes. Following washing, TMB/E substrate was added to effect a color change, and the reaction was quenched. The resulting reaction mixtures were assayed at 450 nm using a microplate reader (μ-Quant; Bio-Tek Instruments, Winooski, VT) with a reference reading made at 570 nm to correct for optical imperfections in the plate. The standard curve was examined for linearity and concentrations of fibronectin were calculated on a computer running KC Junior^{TM}software (Bio-Tek Instruments). The amount of fibronectin in the SIS, SIS_{PAA} and SIS_{EO} was quantified and back-calculated to the amount of starting material in order to yield a measure based on the total dry tissue weight.

2.7 Sulfated GAG content and identification

Total sulfated GAG content and identity (O- versus N- linked) were determined using the Blyscan Sulfated GAG Assay (Biocolor, Biodye Science). Assays were performed in triplicate. Lyophilized SIS, SIS_{PAA} and SIS_{EO}samples (27–39 mg) were added to 20 μl Proteinase K in 180 μl PBS (pH 7.4). Samples were centrifuged at 12, 000 $\times$ g, then heated at 56°C for 45 minutes with intermittent vortexing. To determine the N- versus O-linked GAG content, 100 μl of sodium nitrite solution and 100 μl of 33% acetic acid solution were added to 100 μl of each sample, then vortexed every 5 minutes for 1 hour. Ammonium sulphamate (100 μl) was added and the solution mixed for 10 minutes. Dimethylmethylene blue (1 ml) was added to 100 ml of each sample, vortexed, then mixed on an orbital shaker for 24 hours. Samples were centrifuged at 12,000 $\times$ g for 10 minutes, and the supernatant was removed with care taken to avoid disrupting the GAG-dye complex pellet. The dye was released using 1 ml dissociation reagent (Blyscan) with mixing/vortexing. Absorbance was read at 656 nm–550 nm and GAG concentration was determined using a heparin calibration curve.

2.8 Hyaluronic acid content

Approximately 30 mg of SIS, SIS_{PAA} or SIS_{EO} were incubated overnight at 37°C in 1.125 ml digestion buffer (150 mM NaCl, 6.5 mM KCl, 42 mM $CaCl_2$, 10 mM HEPES, pH 7.0) and 0.375 ml collagenase B solution (Roche, Indianapolis, IN) (final enzyme concentration 0.25 mg/ml). Samples were centrifuged and the supernatant was retained. A 1:40 dilution of each sample in digestion buffer was then incubated overnight at room temperature, and was then assayed using a commercially available ELISA kit for HA (Corgenix, Inc.) according to the instructions provided.

2.9 Scanning electron microscopy

A section of intestine was fixed immediately following slaughter in 3% glutaraldehyde (SEM grade, Ted Pella Inc.) in PBS. Several longitudinal strips (0.5 cm $\times$ 2 cm) of intestine were cut, dipped in liquid nitrogen, and fractured. A hydrated sample of SIS_{PAA} was also fixed in 3% glutaraldehyde. These samples were post-fixed in 1% OsO_4 in 100 mM sodium phosphate buffer for 1 h, serially dehydrated in ethanol (30%, 50%, 70%, 90%, 100%), and critical point dried using liquid CO_2. The samples were mounted such that both the cross section and one of the sides was visible to determine orientation. A sample of SIS_{EO} was hydrated for at least 30 minutes in sterile 0.9% sodium chloride and, using an aluminum foil support, placed upright in a cryo-stage slit holder. Excess water was removed and the samples were plunged into liquid N_2 or placed directly onto the stage in the cryo prechamber and allowed to freeze to -160°C. They were transferred onto the SEM cryostage (at -140°C) and sublimated for 30–45 minutes by raising the stage temperature to -75°C. Samples were sputter-coated with Au for 4 minutes at 1mA current prior to viewing in a JEOL JSM-840 scanning electron microscope using 4–5 kV, 70 μm objective aperture, and probe current of 1, 3 or 6 $\times$ 10^{-11}A. Images were digitally captured using a 160 second dwell time and resolution of 1280 $\times$ 960.

2.10 Statistics

Differences between SIS, SIS_{PAA} and SIS_{EO} were measured for each of the ECM components analyzed using one way analysis of variance (ANOVA) with processing steps as independent variables and matrix components as dependent variables. Significance was set at $p = 0.05$. The Bonferroni method for multiple comparisons was performed to identify significant differences between groups.

Table 1 Growth factor levels at different stages of processing. Values presented represent the amount of growth factor in each gram of tissue

	TGFβ1	VEGF	FGF-2
SIS	4841 ± 193 pg/g	26655 ± 1723 pg/g	49902 ± 1019 pg/g
SIS_{PAA}	892 ± 59 pg/g	159 ± 36 pg/g	105537 ± 6086 pg/g
SIS_{EO}	711 ± 158 pg/g	130 ± 21 pg/g	26736 ± 9687 pg/g

3 Results

3.1 ELISA detection of TGFβ1, VEGF, and FGF-2

Results of the growth factor ELISA assays are presented in Table 1. ELISA results indicated that FGF-2 levels increased significantly in SIS_{PAA}, likely due to removal of the cellular component mass from SIS by the disinfectant. Furthermore, FGF-2 levels remained at more than 50% of the native level in the SIS_{EO}. SIS retained a significant amount of FGF-2 through these disinfection and sterilization processes. The ELISA for activated TGFβ1 indicated that 18% of this growth factor remained in the SIS after PAA disinfection, and that further treatment with EO had no further adverse effect. On the other hand, disinfection with PAA reduced the VEGF content by over 99%, indicating that this growth factor was highly susceptible to this oxidative environment. Taken in total, these results indicate that specific growth factor retention, such as in the case of FGF-2 and TGFβ1, is possible through disinfection and sterilization, but that other growth factors are more susceptible to removal using these methods.

3.2 Fibronectin content

Native, non-treated SIS contained 686 ± 252 ng FN/g dry weight. The majority of the fibronectin (425 ± 123 ng FN/g dry weight in SIS_{PAA} and 415 ± 134 pg FN/g in SIS_{EO}, not significant vs. native SIS) was retained both after disinfection and sterilization. Thus, the level of this important ECM glycoprotein was largely preserved.

3.3 Sulfated GAG content

Total sulfated GAG content (Table 2) was 3.34 ± 0.64 μg/mg dry weight of unprocessed SIS, 12.33 ± 1.93 μg/mg dry weight of SIS_{PAA}, and 10.2 ± 0.8 μg/mg dry weight of SIS_{EO}. *N*-linked GAG accounted for 63, 78, and 74% of the total sulfated GAG content in unprocessed SIS, SIS_{PAA}, and SIS_{EO} respectively. Of note, PAA disinfection significantly increased the amount of detectable sulfated GAG content, likely related to the reduction of the cellular component of the biomaterial following disinfection.

3.4 Hyaluronic acid content

HA is a large structural GAG involved with maintaining the ECM hydration, growth factor binding, and cell signaling [26]. HA analysis indicated the presence of 1990 ± 120 μg HA/g dry weight of native SIS. This level remained essentially unchanged after PAA treatment as well as after EO sterilization (1872 ± 288 μg HA/g dry weight of SIS_{PAA} and 2046 ± 309 μg HA/g in SIS_{EO}, not significant vs. SIS). The retention of the HA through disinfection and sterilization likely contributes to the maintenance of the 3-dimensional structure of SIS through these processes.

3.5 Three-dimensional architecture

Scanning electron microscopy of fixed, cryo-fractured, and critically point dried SIS *in situ* revealed a complex, heterogeneous ECM consisting of fibrous collagen oriented along perpendicular planes, corresponding to the longitudinal and circumferential axes of the small intestine. Figure 1A shows the SIS material situated between the lamina propria, which has fractured to expose the stratum compactum (right), and a thick layer of circular muscle (left). The interface between the lamina propria and the submucosal layer shows no discernable muscularis mucosae. Although the submucosal layer in this region ranges from 60 to 100 μm, plicae within this same preparation approached widths of 300 μm.

Following oxidative disinfection with peracetic acid (Fig. 1B), the isolated SIS_{PAA} lost its cellular and lipid components, resulting in a more homogenous, fibrous scaffold. The removal of these non-collagenous constituents creates voids that are filled by the aqueous surroundings. The perpendicular fiber orientation of the original tissue is preserved. This preparation shows both the open, porous serosal surface (left) and the denser mucosal surface (right). These differences in surface architecture are likely responsible for the differences in scaffold permeability reported elsewhere [27]. A large vascular remnant is present (upper right). Histology has shown these lumen, when arterial in origin, to contain remnants of the internal elastic lamina, the external elastic lamina, or both.

Cryo SEM of rehydrated SIS_{EO} (Fig. 1C) shows the fully hydrated clinically implanted product. While much of the complex architecture of the SIS_{PAA} has collapsed or is embedded within remaining ice, the final SIS_{EO} material maintains the three-dimensional architecture and topographical features of the original SIS tissue.

4 Discussion

Biologic scaffold materials must be safe for clinical use. They must also retain the natural structure and composition of the

Table 2 Sulfated GAG content at different stages of processing. Note that oxidation of the matrix with PAA greatly increases the amount of detectable GAG, and that EO sterilization affects *N*-linked sGAG to a greater extent than it does O-linked species

	N-Linked sGAG	*O*-Linked sGAG	Total sGAG
SIS	2.08 ± 0.5 μg/mg*	1.26 ± 0.2 μg/mg*	3.34 ± 0.64 μg/mg*
SIS_{PAA}	9.63 ± 1.9 μg/mg	2.70 ± 0.04 μg/mg	12.33 ± 1.93 μg/mg
SIS_{EO}	7.50 ± 0.4 μg/mg	2.70 ± 0.3 μg/mg	10.20 ± 0.8 μg/mg

*Statistically lower than SIS_{PAA} and SIS_{EO} ($p < 0.001$) using the Bonferroni method for multiple comparisons.

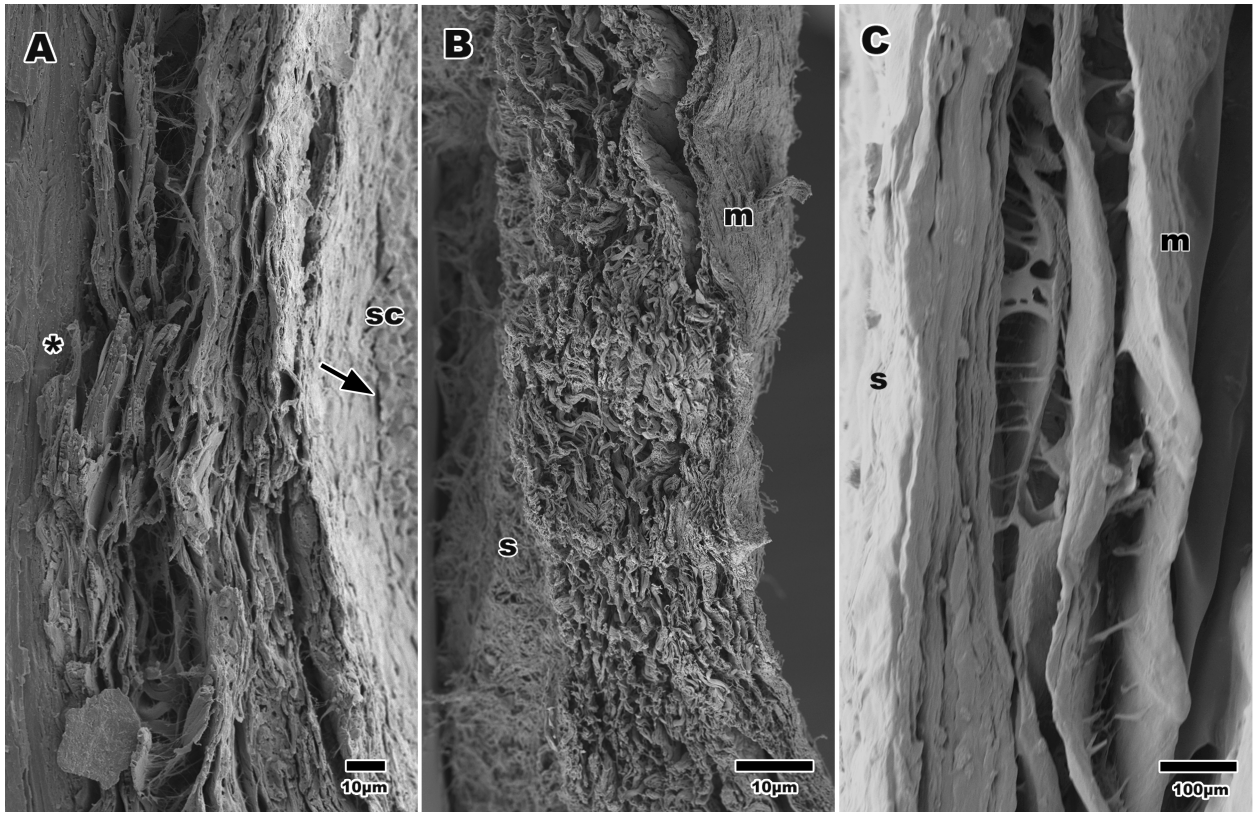

Fig. 1 Scanning electron micrographs of cross-sections of porcine intestine with unprocessed SIS *in situ* (A), SIS_{PAA} (B), and SIS_{EO} (C) at magnifications of 500, 100, and 1000X, respectively. (A) shows the outermost surface of the lamina propria (arrow), which has fractured to expose the underlying stratum compactum (sc) of the tunica mucosa. A thick layer of circular muscle surrounds the submucosa ($_*$). The perpendicular fiber orientation of the original tissue is preserved in SIS_{PAA} (B). This preparation shows both the open, porous serosal surface (s) and the denser mucosal surface (m). A large vascular artifact is present in the upper right corner of the preparation. The complex yet open structure of the native submucosal layer (A) is preserved in the disinfected SIS_{PAA} (B) material, which has undergone an increase in thickness and appears to consist of a more uniform population of fibers. This swelling has abated in the lyophilized, sterilized, and rehydrated SIS_{EO} (C), which has maintained much of the native structure of the extracellular matrix

ECM if they are going to incite meaningful cell and tissue repair and growth following implantation. Data demonstrating the retention of matrix structure and composition following matrix processing into a clinical grade biomaterial is scarce. The retention of growth factors, glycosaminoglycans, and fibronectin in the matrix of a biologic scaffold that has been used effectively in numerous clinical applications, including cutaneous and surgical wound healing applications [15,16,19] was evaluated. We also evaluated the three dimensional architecture of this biomaterial following disinfection with PAA, drying by lyophilization, and terminal sterilization with EO gas.

All matrix scaffolds designed for clinical use that utilize tissues derived from biologic sources must undergo a series of processing steps designed to rid the material of disease-causing agents and provide the sterility required for surgical use. At a minimum, these steps require the use of a disinfectant and a sterilizing agent. PAA has been used to disinfect

biomaterials as it effectively inactivates a wide variety of viruses [28], acts as a local antiseptic agent [29], and inactivates spores and mycoplasms [30]. However, the potential for disruption of the structure and activity of growth factors and other matrix components exists because it sterilizes through oxidation.

Growth factors are known to be relatively labile molecules subject to rapid enzymatic breakdown in their unbound state [31]. The discovery that growth factors are retained in SIS following PAA disinfection suggests that they are sequestered and protected in the matrix in some way. They may be tightly bound to their structural attachment proteins and proteoglycans much like what has been reported for FGF-2 [5]. Two of the three growth factors investigated, FGF-2 and TGFβ1, were retained in the matrix in significant amounts and maintained the structural conformation needed for detection by ELISA. This finding suggests that biological activity specific to each growth factor may also be retained following treatment using PAA. It also supports previously published works using *in vitro* assay techniques [32, 33] showing that the activities of FGF-2 and TGFβ1 are retained in SIS following PAA disinfection and EO sterilization.

FGF-2 and TGFβ1 are better retained in the sterilized tissue than is VEGF. FGF-2 is retained in the ECM tightly bound to heparan sulfate proteoglycans [5]. TGFβ1 is bound in the matrix attached to its latency peptide [reviewed in 34] and HA [35]. The most abundant form of VEGF in SIS, the $VEGF_{165}$ isoform [24], does not tightly bind to the cell surface. It is released into the ECM, exists in its soluble form, and does not tightly bind to ECM proteoglycans [36]. The susceptibility of VEGF to PAA is likely due to its solubility and its inability to tightly bind to other ECM proteins for protection and stabilization.

The levels of total GAG and FGF-2 in SIS_{PAA} were significantly higher than in the non-treated SIS tissue. These finding were unexpected because some loss of these factors due to oxidation was predicted. However, the PAA treatment rids the matrix of unwanted impurities. The apparent increases in FGF-2 and GAG content in SIS_{PAA} are possibly due to the loss of cellular components, such as lipids, nucleic acids, and other undesirable elements naturally present in the non-treated SIS. Additionally, oxidation of collagen by PAA can alter its 3-dimensional structure. This could result in microscopic changes in the matrix structure (Fig. 1) and account for more efficient GAG and growth factor extraction.

The data shows that the absolute amounts of fibronectin and HA are not reduced by PAA treatment. It does not indicate if either of these constituents is otherwise affected by the oxidative treatment. Oxidative damage to fibronectin by peroxides in the presences of reactive metal species is minimal [37], but oxidative damage to HA can be extensive. For example, hydrogen peroxide in the presence of ferrous ions can cause depolymerization of HA through the generation of oxygen free radicals [38]. While it is unclear what effect PAA treatment of SIS may have on the length of the HA polymer, it is hypothesized that differences in the size of HA species lead to different biologic effects [39]. Clearly, further studies to determine the effects, if any, of PAA treatment on HA polymer length are warranted.

Ethylene oxide gas has become a common method for sterilization of biologic tissue implants because other available methods have deleterious effects [40]. EO treatment, however, can alkylate matrix proteins, rendering them inactive and undetectable [41]. The effect of EO sterilization on the growth factor content was therefore examined. TGFβ1 and bone morphogenetic protein were reported to survive EO treatment [6, 42]. All three growth factors examined here, FGF-2, VEGF, and TGFβ1 were retained in the SIS material following EO processing, though FGF-2 content was diminished when compared to the amount present in SIS_{PAA} tissue. As already discussed, the differential susceptibility to EO gas of these growth factors may be the result of the degree of protection afforded by their binding proteins present in the matrix, and the integrity of the glycosaminoglycans to which they are bound. For example, it is likely that the susceptibility of FGF-2 to EO gas is an indirect effect of damage to the heparin chains to which it is bound as a result of PAA oxidation earlier in the purification process.

Lyophilization is a well-established method used to stabilize the structure, bioactivity and composition of proteins, so freeze-drying alone was not expected to alter the growth factor, glycosaminoglycan, or fibronectin content of the SIS. However, it was thought that lyophilization might collapse the three dimensional structure of the matrix and inhibit cell interaction with the surface. Scanning electron microscopy of SIS demonstrated that the submucosal ECM is a complex, micropatterned scaffold consisting of collagenous fibers of various diameters in longitudinal and circumferential orientations. This complexity was greatest in unprocessed SIS and likely due to the heterogeneic tissue composition of the native tissue. The loss of lipids and cells following disinfection with PAA yields a matrix that maintains much of its original topography while yielding greater uniformity in the fiber dimensions. The minor disruption of the underlying matrix by the oxidative process causes a limited swelling in the material that is corrected following lyophilization and EO sterilization. However, the final, clinically used SIS_{EO} material maintains much of the original architecture of the extracellular matrix. These structural features, coupled with bioactive growth factors [32, 33], are likely to be important variables underlying cellular attachment [43], infiltration and eventual incorporation of SIS into healing host tissues.

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