

Influence of pH on Extracellular Matrix Preservation During Lung Decellularization

Running title: Effect of alkalinity on decellularization of the lung

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ABSTRACT 229 words

Abstract

Several studies have shown the utility of using decellularized organs as a substrate onto which appropriate cells can be seeded in an effort to generate bioengineered organs that provide function akin to native tissue. The creation of decellularized organs requires the preservation of the organ extracellular matrix (ECM) as a means to provide critical cues for differentiation and migration of cells that are seeded onto the organ scaffold. The purpose of this study was to assess the influence of varying pH levels on the preservation of key ECM components during the decellularization of rat lungs. Herein, we show that the pH of the 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-based decellularization solution influences the preservation of ECM components of the decellularized rat lung. The preservation of ECM components, including elastin, fibronectin, and laminin, were better retained in the lower pH conditions that were tested (pH ranges tested: 8, 10, 12); glycosaminoglycans were preserved to a higher extent in the lower pH groups as well. The DNA content following decellularization of the rat lung was inversely correlated with the pH of the decellularization solution. These results highlight the effect of pH on the results obtained by organ decellularization and suggest that altering the pH of the solutions used for decellularization may influence the ability of cells to properly differentiate and home to appropriate locations within the scaffold, based on the preservation of key ECM components.

Keywords: Decellularization, Lung, Tissue engineering, ECM (extracellular matrix), DNA

Introduction

The prospect of using decellularized organs that have been recellularized by patient specific progenitor cells for organ and tissue replacement opens the possibility for future clinical applications wherein an essentially autologous transplant occurs¹⁻³. Retention of ECM components within the decellularized organ is crucial in influencing the behaviour of cells that are subsequently placed on the decellularized scaffold⁴⁻⁸. ECM components play a major role in the proper migration, protein expression, and active signalling pathways of the donor cells⁹⁻¹³. Our lab has previously shown that rat lungs decellularized by an alkaline detergent-based decellularization solution retain key extracellular matrix components including collagens, laminin and fibronectin; other matrix components such as elastin and GAGs are significantly diminished^{4,14}. This work also demonstrated that recellularization of these lungs was supported by the remaining ECM. This was demonstrated by reseeding the decellularized lung extracellular matrix scaffold with a heterogeneous pool of neonatal rat lung cells, which appropriately populated the respiratory compartment of the lung with a variety of epithelial cell types, including type 1 and type 2 pneumocytes. While our previous work has shown that ECM components are retained to a detectable level by using a pH 12 CHAPS-based decellularization solution, here we tested the influence of pH on the retention of ECM components.

While an immediate goal of decellularization is to preserve the structure of the lung and its function as a substrate for cell growth, several ECM component proteins are of particular importance because of their abundance in the basement membrane or because of the role that they play in maintaining the mechanical integrity of the organ. For re-population of the decellularized lung extracellular matrix scaffolds, basement membrane proteins such as fibronectins and laminins play a direct role in the appropriate attachment and differentiation of seeded cells^{8,9}. For the maintenance of tissue architecture and to ultimately support breathing,

critical ECM components including collagens, elastin, and proteoglycans are required⁸. Retention of both the integrity of the basement membrane and mechanical function must be taken into account for optimization of the tissue engineering process.

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We have previously compared two detergent-based methods of lung decellularization, one based on CHAPS (8mM in PBS with 1M NaCl and 25mM EDTA) and the other based on sodium dodecyl sulphate (SDS; 1.8mM SDS in PBS with 1M NaCl and 25mM EDTA)¹⁴. These findings indicated that decellularization with 8mM CHAPS resulted in better collagen
100 retention and, as a consequence, produced lungs with greater mechanical integrity when compared to the 1.8mM SDS-based decellularization procedure. Both methods of decellularization, however, resulted in large losses of other ECM components including loss of elastin and sulfated glycosaminoglycans. Other methods of organ decellularization include the use of chemical methods that rely on alkaline conditions to decellularize tissues and organs
105 as a means to disrupt cellular membranes and remove nucleic acids^{2,15}. Given that the pH of the decellularization solution plays a role in the loss or retention of ECM components in other tissues, notably pericardium¹⁶, we tested various CHAPS-based decellularization solutions varying only in pH in an effort to understand what role pH plays in the maintenance of the lung ECM. CHAPS solutions ranged in pH from 8 to 12. We found that lungs decellularized with
110 the lower pH solutions retained more elastic fibers, fibronectin and laminin. Glycosaminoglycans (GAGs) were also retained to a greater extent in the more neutral pH ranges. Histological analysis also confirms that the more mild pH decellularization conditions favour better preservation of the ECM. However, while DNA content was significantly decreased in all pH groups tested, there was more DNA retained in the lungs within the lower
115 pH groups when compared to the pH 12 condition. Taken together, these data implicate the importance of pH levels on the preservation of the ECM.

Materials and Methods

Organ Harvest and Decellularization

120 Lung tissue was obtained from young adult (3-month-old) male Sprague Dawley rats (Charles River, Wilmington, MA). All animal work was performed with approval from the Yale University Institutional Animal Care and Use Committee, and all animal care complied with the Guide for the Care and Use of Laboratory Animals. Organ harvest was performed as previously described ⁴. Briefly, animals were euthanized via intraperitoneal injection of sodium
125 pentobarbital (140 mg/kg, Sigma-Aldrich Inc, St. Louis, MO) and heparin (250U/kg, Sigma). The lungs were perfused via the right ventricle with PBS containing 50 U/ml heparin (Sigma) and 1 µg/ml sodium nitroprusside (Fluka) and the heart, lungs and trachea were dissected and removed en bloc. Tracheal and pulmonary artery cannulae were inserted and sutured into place, to provide access for perfusion and decellularization. Per our previously published methods,
130 lungs were decellularized via perfusion of the pulmonary artery while maintaining physiologically appropriate pulmonary arterial pressures (i.e., below 25 mmHg) ⁴. In each case, a total of 500 ml of decellularization solution (8 mM CHAPS, 1 M NaCl, 25 mM EDTA in 1X PBS) was infused via the pulmonary artery; pH was adjusted prior to decellularization using hydrochloric acid to final pH values of 8.0, 10.0, and 12.0

135 After decellularization, tissues were extensively rinsed via vascular perfusion with 1X PBS. The decellularized tissues were further rinsed with PBS containing 10% FBS, 10% pen-strep solution and 2% gentamicin for 48 hours in order to remove any remnant cellular debris as described previously ¹⁷.

Histological Analysis and Immunohistochemistry

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Tissues were formalin-fixed, paraffin-embedded and sectioned at a thickness of 5 μ m. Analysis was performed with standard hematoxylin and eosin staining, elastic van Gieson (for elastin), and Alcian blue at pH 2.5 (for proteoglycans), as well as staining for DNA using 4', 6-diamidino-2-phenylindole (DAPI). For immunohistochemistry, tissue sections were deparaffinized, rehydrated, and rinsed in PBS with 0.2% Triton X-100 for 15 minutes. Antigen retrieval was performed with 20ng/ml proteinase K in TE buffer (pH 8.0), at 37°C for 10 minutes. Sections were then blocked with 5% BSA and 0.75% glycine in PBS for 1 hour at room temperature. Primary antibodies for fibronectin and laminin were applied 1:100 in blocking buffer overnight at 4°C, followed by secondary antibodies at 1:500 dilution for 1 hour at room temperature. Primary antibody for β -actin was applied at 1:200 in blocking buffer. Secondary antibodies used were mouse anti-rat antibody. Slides were mounted using DAPI-containing mounting media.

Western Blot

Tissues were digested in RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% [v/v] Triton X-100, 0.5% [w/v] sodium deoxycholate, and 0.1% [w/v] SDS) with freshly added protease inhibitors and homogenized at 15,000 rpm for 30 seconds. After incubation for 1 hour at 4°C, insoluble particles were removed by centrifugation at 14,000g for 25 minutes. Protein concentration was quantified via Bradford assay, and then placed in Laemmli's reducing buffer for 25 minutes at 65°C. Protein was run on polyacrylamide gels, transferred to a nitrocellulose membrane, and blocked for 2 hours in 5% non-fat dry milk (NFDM) in TBS with 0.05% tween-20 (TBS-T). Primary antibodies of monoclonal anti-rat β -actin at a dilution of 1:2000 and monoclonal anti-rat MHC class I antibody of RT1A at a dilution of 1:500 were applied overnight in 1.5% NFDM in TBS-T, followed by horseradish peroxidase-conjugated goat

165 secondary antibodies for 1 hour at room temperature at a dilution of 1:2000. Protein was detected using substrate from Supersignal West Pico.

Collagen Assay

Collagen was quantified with a colorimetric assay to detect hydroxyproline using a
170 modification of Grant's method¹⁸. Lung samples were lyophilized and weighed, then incubated in papain (10U/ml; 25 mg/ml) at 60 °C overnight (Sigma). Papain-digested samples were incubated in 6 N HCl at 115 °C for 18 h, neutralized, oxidized with chloramine-T and then reacted with p-dimethylaminobenzaldehyde. Absorbance was measured at a wavelength of 550 nm and a 1: 10 w/w ratio of hydroxyproline to collagen was used to calculate the collagen
175 content of the tissue.

Sulfated GAG Assay

Sulfated GAGs were quantified using the Blyscan GAG assay kit (Biocolor). Lung samples were lyophilized and weighed, then incubated in papain (25 mg/ml) at 60 °C overnight (Sigma).
180 Papain-digested samples (prepared as described for the collagen assay, above) were assayed according to the manufacturer's instructions. Absorption was measured at a wavelength of 650 nm, and GAG content was quantified using a standard curve.

DNA Assay

185 DNA content of tissues was quantified using the Quant-iT PicoGreen dsDNA assay kit, following the manufacturer's instructions. Briefly, tissue samples were weighed and lyophilized, diluted in TE buffer and mixed with the Quant-iT PicoGreen reagent. Fluorescence was measured at 535 nm with excitation at 485 nm, and DNA content was quantified using a standard curve.

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Transmission Electron Microscopy (TEM)

Samples were fixed using 4% paraformaldehyde in PBS and then placed in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1M sodium cacodylate buffered fixative for 2 hours at room temperature. The samples were rinsed in 0.1M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 hour, then stained en bloc in 2% uranyl acetate in maleate buffer pH 5.2 for a further hour. The samples were rinsed, dehydrated through a graded ethanol series and infiltrated with epon resin and baked overnight at 60°C. Hardened blocks were cut using a Leica UltraCut UCT and 60 nm sections were collected on nickel grids and stained using 2% uranyl acetate and lead citrate. Samples were viewed on a FEI Tencai Biotwin TEM at 80kV. Images were taken using a Morada CCD digital camera using iTEM (Olympus) software.

Results

Effectiveness of Decellularization is Dependent on pH

The gross morphological appearance of lungs decellularized at pH 12 differed from native tissue in that they were smaller, white and semi-translucent. Lungs decellularized at pH 8 and at pH 10 appeared discoloured (brown) and slightly less translucent than those decellularized at pH 12 (data not shown). Nevertheless, all lungs were completely devoid of intact cells by standard hematoxylin and eosin histological staining (Fig. 1B, C and D), though some deep blue remnants (DNA smears) were occasionally noted throughout the lung. These smears of DNA were more prevalent in the pH 8 group than in the pH 10 or 12 groups (Fig. 1B). Additionally, though all decellularized lungs retained the overall structure of both the proximal and distal regions of the lung, the alveolar septae appeared to be more intact in the lower (i.e., pH 8, 10) rather than higher (i.e., pH 12) group, (Fig. 1B and C, compared to D) suggesting more damage to the matrix in the higher pH group.

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Immunostaining for β -actin demonstrated an increase in the effectiveness of cytoskeletal protein removal with increasing pH. The alveolar septae remained strongly positive in the pH 8 group when compared to native tissue (Fig. 1B and 1A, respectively). Under high power magnification, a few cells and residual cytoskeletal debris was scattered throughout the alveolar septa in the pH 8 group (Fig. 1F). In the pH 10 group, the β -actin staining of the alveolar septae was relatively weak (Fig. 1G). In the pH 12 group, the majority of the lung tissue was negative for β -actin staining (Fig. 1H); the alveolar septae were immune-negative for β -actin and denatured cell components were not evident (Fig. 1H). Western blot analysis revealed that MHC class I was not detected in any of the decellularized groups (Fig. 1I). However, beta actin was detected in the pH 8 group and weakly in the pH 10 group, suggesting insufficient decellularization when using a CHAPS-based solution at lower pH (Fig. 1I).

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When quantifying DNA by the Quant-iT PicoGreen assay, it appears that the DNA content of all decellularized scaffolds were significantly lower than that of native lung ($p < 0.0001$) (Fig. 1J). However, the contents were significantly higher in the pH 8 and pH 10 groups than that in pH 12 group ($p < 0.001$). Surprisingly, the DNA contents per tissue weight were similar in pH 8 and pH 10 groups, and native lung (Fig. 1K).

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Protein Content and Organization after Decellularization is pH Dependent

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Verhoff von-Gieson (VVG) staining was utilized to evaluate the presence and organization of elastic fibers, a critical component for elastic recoil of lung tissue. Using this stain, it was determined that the septae of lower pH groups appeared thicker and possessed a greater abundance of dark, wavy elastic fibers (in black) than those of the higher pH groups (Fig. 3B, compared to 3C-D). Elastin content appeared to be less than that of native lung, however (Fig.

3A). Identifiable fibers were largely absent in the lungs decellularized at pH 12. These data suggest that elastic fibers are only preserved when using decellularization solution at pH groups lower than 12.

In order to evaluate the presence of GAGs, Alcian blue histologic staining was utilized. One or more GAG chains link to a core protein of proteoglycans, and by sequestering water contributes to the “viscous component” of lung viscoelasticity which plays a fundamental role in protecting lung mechanical integrity. GAGs were noticeably diminished compared to native lung in decellularized tissues, especially in the groups decellularized at pH 10 and pH 12 (Fig. 2C-D).

Immunohistochemical staining of fibronectin revealed that large blood vessels remained immunopositive for fibronectin after decellularization in both of the low pH groups, but that fibronectin was largely absent in the high pH group (Fig 3B-D). With respect to the airways, the alveolar septa were strongly immunopositive for fibronectin in the pH 8 group and greatly diminished in the pH 10 and 12 groups (data not shown). Laminin was also strongly positive in the alveolar septa in both the low pH groups, but was markedly reduced in the high pH group (Fig 3F-H).

Collagen, Sulfated GAG and DNA Contents are Affected by pH

A hydroxyproline collagen assay showed that total collagen content was diminished by high pH. Although the content was significantly lower in pH 10 and pH 12 groups than that of native lung ($p < 0.05$), the remaining collagen was 70% of native lung, whereas the pH 8 group not significantly less than native lung (Fig. 2E).

Based on the use of a kit for the detection of sulphated GAGs, the GAG content of the decellularized scaffolds was significantly lower than that of native lung ($p < 0.005$; suffering a loss of nearly 80% in the pH 8 and pH 10 groups and 90 % when decellularized at pH 12 (Fig. 2F). No quantitative difference was observed between lungs decellularized at pH 8 or 10.

Transmission electron microscopy

Decellularization of lungs at pH 8, 10 and 12 resulted in the removal of intact cells and preservation of alveolar architecture and capillary structures (Fig. 4B-D), though the presence of cell debris appears to be inversely correlated with pH, as cellular remnants appear at pH 8 and 10, but not 12 (Fig. 4B, C, compared to D). In all cases, separation of compartments was maintained by intact basement membranes. The presence of collagen fibrils within the septae further illustrates the structural integrities of the decellularized scaffolds.

Discussion

Matrix scaffolds fabricated from decellularized donor organs provide a means to tissue engineer custom-tailored and fully functional tissue for use in regenerative medicine. The goal of the present study was to further optimize our previously established CHAPS-based decellularization process by adjusting the pH of the solution to minimize damage to the ECM scaffold, while maintaining the effective removal of cellular and nuclear material. Towards this goal, we examined the effectiveness of CHAPS as a decellularization agent at three different pH levels while examining the retention and organization of collagen, elastin, GAGs, laminin and fibronectin in lung tissue. We found that decreasing the pH of the CHAPS solution resulted in a dramatic increase in global matrix preservation; however, this decrease in detergent alkalinity also resulted in a concomitant decrease in decellularization efficiency. Furthermore, these results demonstrate that CHAPS-based decellularization at all pH levels chosen is very

effective at lysing cells and removing cell membrane components, but complete removal of
DNA and cytoskeletal components required higher pH levels (pH 12).

When examining the gross morphological consequence of CHAPS-based decellularization, lungs exposed to detergents at pH 12 decreased in volume and resulted in a viscous effluent. In the lower pH groups (pH 8 and pH 10), the lung appeared very swollen; the entirety of the lung was filled with excessively viscous liquid. Interestingly, the observed swelling was still present in lungs treated with decellularization solution at pH 11 (data not shown).

In terms of decellularization effectiveness, immunoblotting showed that MHC class I is negative in all decellularized groups (at all pHs) indicating that the components of 8mM CHAPS, 1M NaCl, and 25mM EDTA are sufficient to lyse cells and remove cell membranes at all pH values examined. However, β -actin was detected via immunoblot and immunofluorescence in the group decellularized at pH 8, suggesting that CHAPS-based cytoskeleton removal is compromised at a lower pH. Further, when examining total DNA content (FIGURE 1I, J), the removal of DNA was significantly decreased at lower pHs, compared to higher pHs. Taken together, these data indicate that decellularization by the solution described is partially compromised when utilized at lower pHs.

The observed difference in lung distension and effective decellularization between groups is likely due, in part, to pH-dependent swelling of liberated DNA¹⁹. Specifically, the viscosity of DNA is very high at lower pHs (below 11.7), and at pH 11.7 is dramatically reduced to 10.9% of the intrinsic viscosity, and finally plateaus around a pH of 12²⁰. At pH 12, DNA denatures to a single strand (each single strand is free to adopt different random coiled conformations), each with a lower intrinsic viscosity than the double stranded DNA.

Therefore, it is possible that insufficient DNA removal observed in lower pHs might simply be
 315 due to the combination of high viscosity of DNA and the complex architecture of a whole organ.
 When using the CHAPS-based protocol described by our group, therefore, the “handlability”
 of the organ that is conferred by high pH may outweigh the gains in matrix retention that is
 observed with lower pH solutions.

320 In our previous studies, we determined that CHAPS-based decellularization (at pH 12) resulted
 in decreased GAG and elastin content in decellularized tissues but not in total collagen content
¹⁴. GAGs are found on cell surfaces, within intracellular vesicles, and incorporated into the
 ECM ²². They help control macromolecular and cellular movement across the basal lamina,
 bind growth factors and cytokines, and contribute to the properties of the ECM by creating
 325 repulsive forces to sequester water using negatively charged ‘tails’ ²³. Because GAGS are
 intrinsically part of the cell surface, removal of cells and cell components will ultimately result
 in the depletion of cell-bound GAGs. Additionally, the solubility of GAGs embedded within
 the ECM increases in alkaline solutions ². Accordingly, GAG levels were significantly lower
 in the higher pH groups (as evident by histology and the quantitative GAG assay) in the
 330 experiments described herein. However, despite the decrease in GAG content, we have
 previously demonstrated via stress-strain curves that our CHAPS-treated decellularized
 scaffold retains an ultimate tensile stress similar to normal lung ¹⁴.

Elastin is also an important ECM component in lung parenchyma, as it contributes to tissue
 335 elasticity, extensibility, and the intrinsic tissue recoil property essential for breathing ²⁴.
 Additionally, elastic fibers endow the pulmonary vasculature with resilience and are critical to
 the function of arteries ²⁵. Because of the low post-natal production and slow turnover of elastin,
 de novo elastin formation is difficult to achieve. In addition, without sufficient elastin content,

tissue degradation and calcification could occur upon implantation ²⁶. Therefore, it would be highly desirable to preserve elastin during decellularization. In the present study, elastic fibers were more abundantly observed in lower pH groups than the high pH group, indicating a pH dependent preservation of the matrix.

Fibronectin and laminin are both important ECM basement membrane proteins for cell adhesion. They serve a prominent role in the normal biological function and development of the lung and promote the formation and maintenance of the vasculature ¹². As evidenced by histological examination, using the decellularization solution at a lower pH preserves the majority of the vascular and alveolar basement membranes. Specifically, immunostaining for fibronectin clearly showed fibronectin positive structures throughout the alveoli and in the vessels of the lungs decellularized at pH 8. The blood vessels retained fibronectin at pH 10 but these structures were immunonegative at pH 12. The matrix retention exhibited at lower pHs may provide an additional advantage for cell attachment and adhesion in the recellularization process.

The use of CHAPS under alkaline conditions might be a “double-edged sword”: it allows for complete removal of cells and cellular debris, but in exchange for substantial matrix damage including loss of GAGs, elastin, fibronectin, and laminin. If the goal of decellularization is total removal of DNA and immunogenic molecules (i.e., prioritizing removing donor immunogenicity) ²⁷, it seems that higher alkaline treatment detergent would be a better choice as a decellularization solution. Macchiarini and colleagues reported that although decellularized tissue-engineered trachea still retained some pre-existing cellular elements in the cartilaginous regions, it did not induce inflammation and did not require immunosuppressive drugs. They speculate that retained elements may even provide helpful

signals to both graft and host cells³. However, the preservation of key matrix components is
365 important for successful clinical use to restore functionality to the organ of interest. Therefore,
our data indicate that a mid-range, reduced pH CHAPS-based solution would be a suitable
compromise (e.g., at pH 10). Further evaluation may be beneficial in order to clarify which pH
is most suitable for generating an appropriate decellularized extracellular matrix for clinical
use.

370 **Conclusions**

This study contributes to the growing body of literature dedicated to producing a decellularized
scaffold for use in regenerative medicine. Our comparison of the decellularization capacity of
a CHAPS-based decellularization solution at different pHs clearly shows differential effects in
375 terms of matrix preservation and cellular removal. The lower pH solution suppressed ECM
damage, most notably the loss of GAG and elastin content, but could not effectively remove
all DNA. Although recellularization and eventual clinical effect remains to be seen, the present
pH-based study adds important information in the field of tissue engineering and scaffold
generation via decellularization.

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Author Disclosure Statements

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Figure Legends

Fig. 1. Effectiveness of CHAPS-based decellularization is pH dependent.

Hematoxylin and eosin (A-D) and β -actin immunostaining (E-H, in brown) of lungs
460 decellularized at various pHs. When compared to native lung tissue (A), no intact
nuclei are observed in any decellularized tissue (B-D). There are however substantial
DNA remnants in lung decellularized at pH 8 (B, E), and to a much lesser extent in
when decelled at pH 10 (C, G). Most of the cell DNA and cytoskeletal staining is
negative in pH 12 group (D, H). Cell membrane protein MHC class I is not detected in
465 any decellularized groups (I), and β -actin is only detected in the native and pH 8
group. DNA quantification in native and decellularized lungs (J, K) indicates that DNA
decreases with increasing pH of decellularization solution. Western blot analysis for
MHC class I and β -actin in decellularized lungs. Scale bar = 100 μ m. Asterisk
indicates significantly difference between the groups (*; $p < 0.0001$, **; $p < 0.01$)
470 Data are expressed as mean values \pm SD, $n = 5$.

Fig. 2. Retention of GAGs and collagen in decellularized lungs is pH dependent.

Immunostaining for GAGs (A-D) in lungs decellularized at various pHs. Alcian blue
staining reveals alveolar septae are strongly positive for GAGs in native lung (A), and
475 dramatically decrease in GAG content when treated with increasing pH of decellular
solution. Collagen and sulfated GAG quantification (E,F) in native and decellularized
lungs. Scale bar = 100 μ m. Asterisk indicates a significant difference between the
groups (*; $p < 0.05$, **; $p < 0.005$). Data are expressed as mean values \pm SD, $n = 5$.

Fig. 3. Maintenance of elastin, laminin, and fibronectin in decellularized lungs is pH dependent. Elastic-Van Gieson (EVG) staining for elastin shows highly concentrated elastic fibers in native lung (A), reduced amounts in pH 8 group (B) and severely diminished levels in the pH 10 and 12 groups (C-D). Alveolar septae are strongly positive for laminin in native lung (E). Laminin and Fibronectin are generally preserved in pH 8 and pH 10 groups (F and G; J and K), but not in pH 12 group (H, L). Scale bar = 100 μ m.

Fig. 4. Transmission electron microscopy demonstrates maintenance of alveolar architecture and intact alveolar-capillary barriers. Native (A) and decellularized tissue at pH 8 (B), 10 (C) and 12 (D) illustrate maintenance of basement membranes (arrows) and overall alveolar architecture. Distinctions can be made between air spaces (A) and capillaries (C); collagen fibrils are present in the alveolar septae (arrow heads, black \blacktriangleright). While some cell debris is noted at pH 8 and 10 (double arrow head, \gg), pH 12 is devoid of cellular components. Scale bar in A =5 μ m, B=1 μ m, C=2 μ m, D=5 μ m.

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Fig. 1.

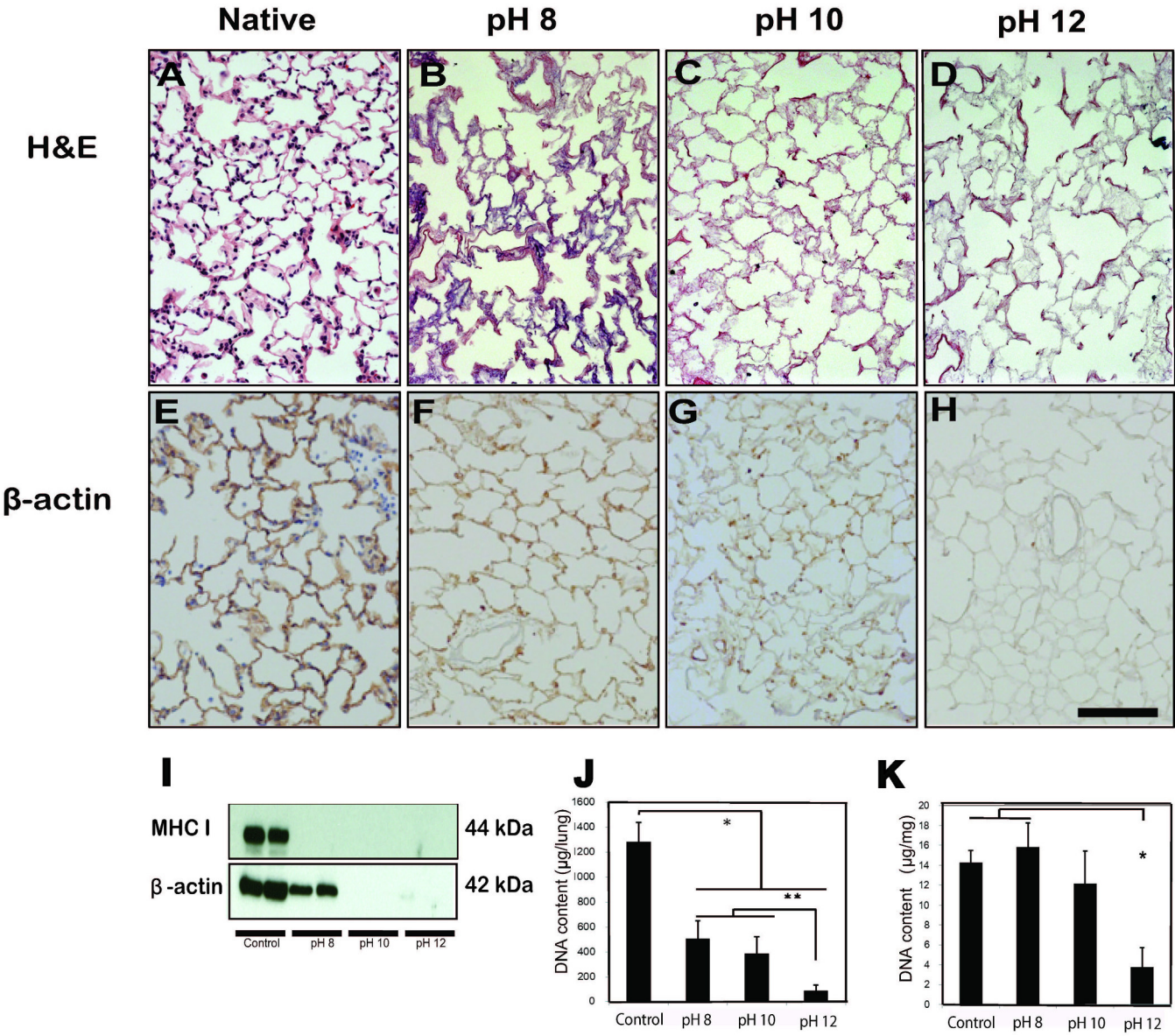


Fig. 2.

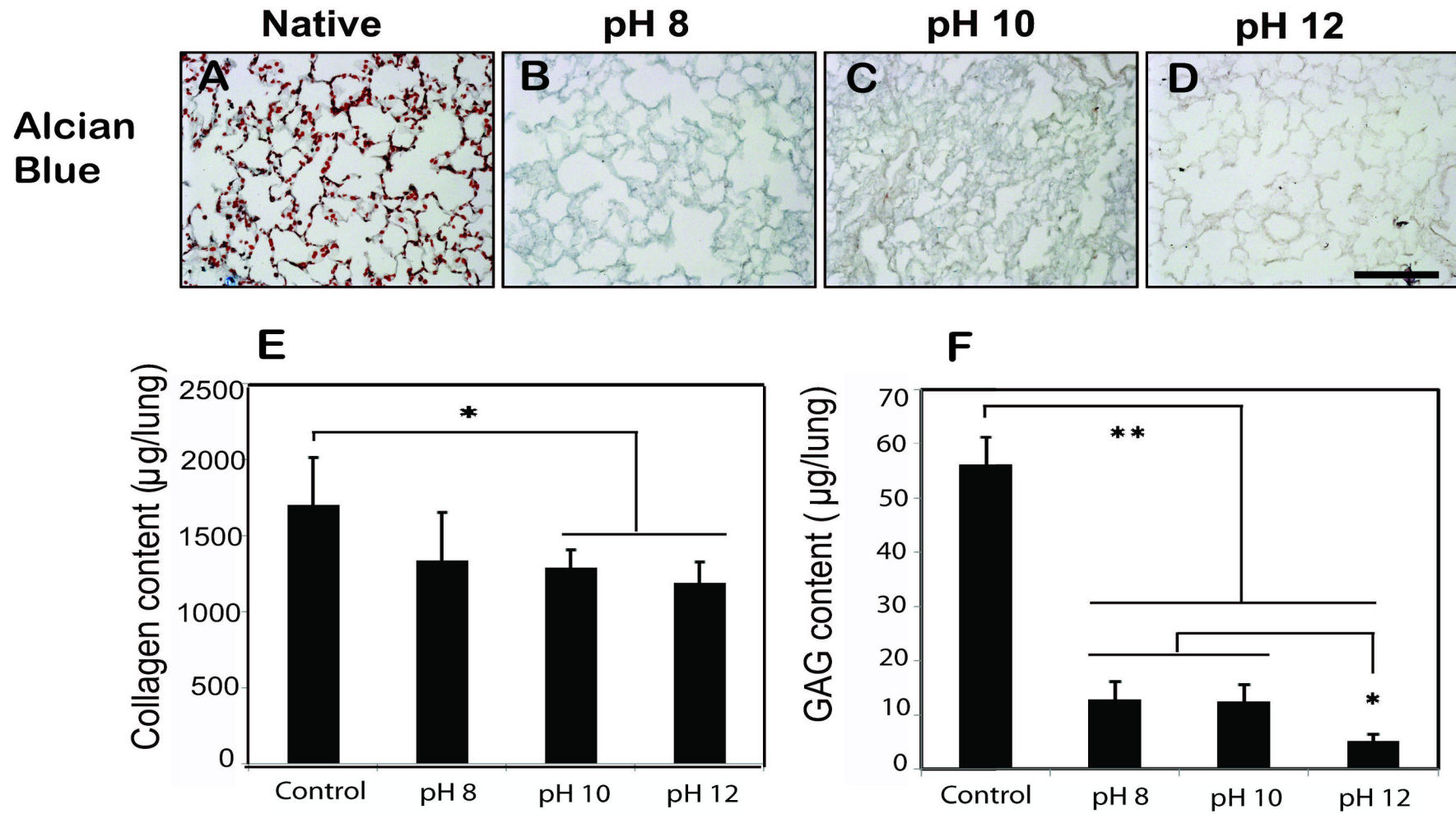


Fig. 3.

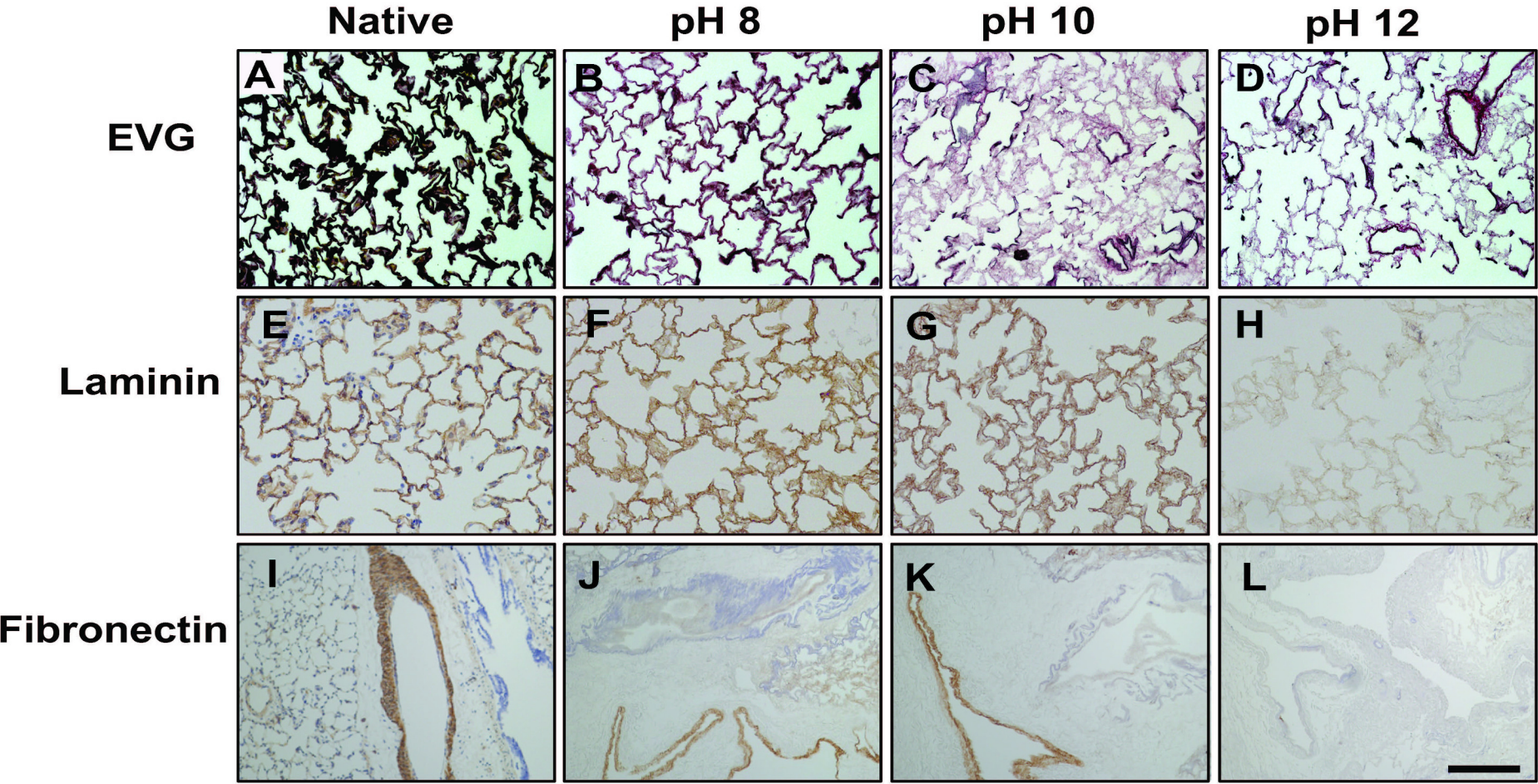


Fig. 4.

