


Rat Bile Duct Decellularization

Silvia Baiguera¹  · Svetlana Arkhipva² · Dengping Yin³ · Mark Holterman⁴ · Paolo Macchiarini²

Published online: 28 September 2016
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Abstract The repair and reconstruction of bile ducts damaged by disease or trauma remains a vexing medical problem. In particular, surgeons have few options when it comes to a long segment reconstruction of the bile duct. Tissue-engineered substitutes, with properties similar to the native tissue, might represent a solution for the functional reconstruction of bile ducts. In particular, decellularized tissues and organs represent a suitable option for tissue engineering when specific scaffolds are needed. However, the optimal conditions to completely remove all the cellular components and minimally affect the structural and residual biochemical properties of the extracellular matrix are still to be found. This paper presents the characterization of rat bile ducts after implementing an established detergent-enzymatic decellularization method. One cycle was enough to generate a complete decellularized bile duct matrix, histologically and structurally similar to the native one. The network of collagen, reticular and elastic fibers, found in the native bile duct matrix was well preserved. Moreover, the decellularization approach did not affect the elastin content.

Keywords Decellularization · Detergent-enzymatic approach · Bile ducts · Extracellular matrix

1 Introduction

Iatrogenic and traumatic bile duct injuries, atresia, and sclerosis are currently treated with invasive procedures that, even when made by expert surgeons, can be associated with high mortality and morbidity [1, 2]. Plastic or metal stents are currently used for biliary tract reconstruction but these invasive approaches are often associated with severe side effects, including chronic cholangitis and the development of secondary strictures and may require multiple interventions. The development of new methods for effective reconstruction of the biliary tract is a highly important task. The total or partial substitution of the bile duct with a bioengineered graft could represent an innovative and alternative approach to the classic surgical procedures to reestablish the physiological biliary-digestive continuity.

Biological scaffolds, composed of extracellular matrix (ECM), have recently been particularly interesting for researchers because of their unique properties, such as the conservation of the natural ECM composition, production of non-toxic biodegradable products, capability to avoid inflammation, and their natural degradability by cellular enzymatic activity, with release of growth factors and peptides that could stimulate constructive tissue remodeling [3, 4]. Previously, we described an effective detergent-enzymatic method (DEM) to obtain bioengineered matrices from a variety of tissues and organs [5–9]. Here, we optimized the DEM approach to obtain a bile duct acellular matrix devoid of cellular components while still

✉ Paolo Macchiarini
PMacchiarini@kpfu.ru

¹ BIOAIRLab, University Hospital Careggi, Florence, Italy
² Laboratory of Bioengineering and Regenerative Medicine (BioReM), Kazan (Volga Region) Federal University, Kazan, Russia
³ Animal Microsurgery Center, Departments of Surgery, Biological Science Division, University of Chicago, Chicago, IL, USA
⁴ University of Illinois College of Medicine Peoria, Illinois, USA

retaining the micro- and ultra-structural native architectural details of the bile duct matrix.

2 Materials and Methods

2.1 Materials

Phosphate-buffered saline (PBS), deoxycholate, DNAsi, and antibiotic and antimycotic solution were supplied by Sigma-Aldrich (Milan, Italy), while paraffin, glutaraldehyde, and hematoxylin and eosin (H&E) by Merck (Darmstadt, Germany). Movat pentachromic stain kit was supplied by Diapath (Bergamo, Italy), 4'-6-diamidino-2-phenylindole (DAPI) by Vector Laboratories (CA, USA), and sodium cacodylate buffer (pH 7.2) by Prolabo (Paris, France). Fastin™ elastin assay kit was provided from Biocolor (Carrickfergus, UK). All materials and reagents were used as received.

2.2 Study Design

Male Brown Norway rats ($n = 15$) (Charles River Laboratories Italia S.r.l., Calco, Italy), weighing 230–320 g, were used as bile duct donors. All animals received care in compliance with the “Principles of laboratory animal care” formulated by the National Society for Medical Research and the “Guide for the care and use of laboratory animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care and Use Committee and the Bioethics Committee of the University of Florence (Italy). Rats were individually housed and maintained at an environmental temperature of 25 ± 2 °C and on a 12/12 h light/dark cycle. Animals were acclimated for 7 days before experiments.

Bile ducts ($n = 15$) were used for structural (H&E and Movat staining), morphologic (scanning electron microscopy, SEM), matrix content (elastin quantification), and effectiveness of decellularization (DAPI staining) evaluation.

2.3 Decellularization Process

After harvesting, bile ducts were stored in cold PBS, containing 1 % antibiotic and antimycotic solution, and subsequently treated using a modification of the DEM, already used for different tissues and organs decellularization [5, 7–11]. Briefly, samples were processed as follows: osmotic lysis (distilled water), detergent cell-extraction (sodium deoxycholate, 4 %), DNA digestion (DNAsi, 2000 KU DNAsiin1 M NaCl), and washing (distilled water) steps. All the decellularization process was performed using agitated baths at 60 rpm. After washing step, bile duct samples were stored in PBS containing 1 % antibiotic and antimycotic solution at 4 °C. Different time

periods of each treating steps, mainly osmotic lysis and detergent cell-extraction, were evaluated to obtain a complete tissue decellularization without losing much of the bile duct matrix. To evaluate the decellularization process, aliquots were retrieved and analyzed by H&E and Movat staining.

2.4 Decellularized Bile Duct Characterization

2.4.1 Histological Analysis

Parts of bile duct samples (native and decellularized) were fixed for 24 h in 10 % neutral-buffered formalin solution in PBS (pH 7.4) at room temperature. They were washed in distilled water, dehydrated in graded alcohol, embedded in paraffin, and sectioned at 5 μ m thickness. Adjacent sections were deparaffinized, rehydrated, and stained with H&E to evaluate tissue decellularization and morphology. To evaluate tissue morphology, each sample was also stained with the Movat pentachromic stain kit, according to the manufacturer’s protocols.

2.4.2 Assessment of Cellular Content

To evaluate the remaining cells after DEM, adjacent sections (5 μ m thickness) were deparaffinized, rehydrated, and stained with DAPI, a fluorescent nucleic acid stain (VECTASHIELD Mounting Medium with DAPI; excitation wavelength 350 nm, emission wavelength 460 nm) for 30 min at room temperature in darkness, and analyzed by fluorescence microscopy.

2.4.3 Morphological Characterization

To qualitatively evaluate the decellularized matrix structure, bile duct (native and decellularized) matrices were fixed with 3 % (v/v) glutaraldehyde in a buffered solution of 0.1 M sodium cacodylate buffer (pH 7.2). After rinsing in cacodylate buffer, specimens were dehydrated through an ethanol gradient, critical point dried, sputter coated with gold, and observed by means of scanning electron microscopy (SEM; JCM-5000 NeoScope, Nikon).

2.4.4 Elastin Content Measurement

Insoluble elastin was extracted from native and decellularized samples ($n = 4$ for each condition) as soluble cross-linked polypeptide elastin fragments, using the hot oxalic acid extraction technique. Wet samples were mixed with oxalic acid (0.25 M) and boiled in a water bath for 1 h. The supernatant was collected by centrifugation, and the sediment was submitted to a second and third extraction under the same conditions. Soluble elastin content in the oxalic extracts was determined using the colorimetric Fastin™ elastin assay kit, based on a fastin dye reagent (5,10,15,20-tetraphenyl-21,23-porphine

tetrasulfonate), following the manufacturer's instructions. Briefly, samples were added with elastin precipitating reagent, incubated for 15 min, and centrifuged. The dye reagent was then added to allow the formation of elastin-dye complex.

After incubation (90 min) and centrifugation, the elastin-dye complex was dissolved by incubation with the dye dissociation reagent for 10 min. Absorbance was measured at 513 nm on an Epoch Microplate Spectrophotometer (BioTek, VT,

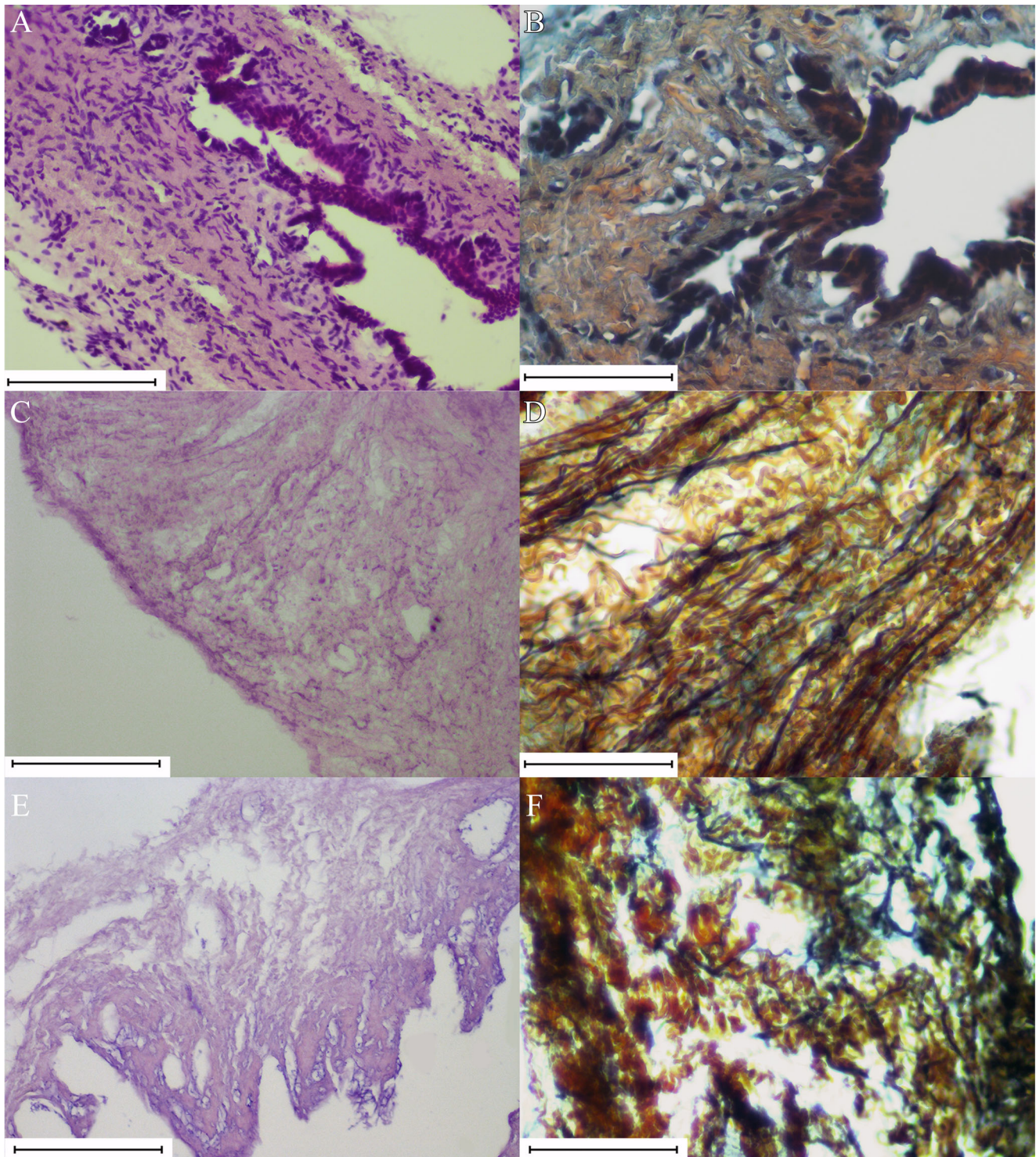


Fig. 1 Hematoxylin and eosin (a, c, e) and Movat pentachromic (b, d, f) staining of native bile ducts (a, b) and of bile ducts (c–f) after one decellularization cycle (water step: 1 h (c, d); 18 hr (e, f)).

Black indicates nuclei and elastic fibers, *red* smooth muscle, while *yellow* collagen and reticulum fibers (a, c, e: scale bar = 100 μm; b, d, f: scale bar = 200 μm)

USA). Replicate samples were averaged and corrected by subtracting the blank average, and elastin content was determined from a standard curve constructed using five concentrations (5–25 mg) of α -elastin. Final values were expressed as milligrams of elastin per wet weight.

2.5 Statistics

Results are expressed as mean \pm standard deviation. Significant differences were estimated by Mann-Whitney *U* test. *p* values less than 0.05 were considered significant.

3 Results

Bile ducts were firstly processed with one DEM cycle using the following time periods: distilled water (1 h or 18 h), deoxycholate (2 h), water (15 min), DNAsi (60 min), and water (15 min).

As shown in Fig. 1, the surface epithelium of native bile ducts is a single layer of tall, uniform, columnar cells and the mucosa forms irregular pleats and small longitudinal folds. The subepithelial region is made of hypocellular connective tissue containing elastic and few smooth muscle fibers, few mucous glands, fibroblasts, lymphoid cells, and few leukocytes (Fig. 1a). The muscularis externa is not well defined and consists of an incomplete thin layer of scattered bundles of smooth muscle fibers, circularly arranged, running in the longitudinal and oblique direction (Fig. 1b). Adventitia is made of loose connective tissue. After one decellularization step, almost all cellular elements were removed (Fig. 1c). As Movat pentachromin staining showed, many elastic, reticular, and collagen fibers were visualized. However, some matrix damages were observed. Furthermore, treating bile ducts for 18 h with water did not enhance the decellularization process, and led to severe matrix degradation (Fig. 1c–f). Movat pentachromin staining showed the absence of framed elastic fibers, degenerated connective fibers, and, in many samples, their aggregations were loose with numerous cavities in between (Fig. 1f).

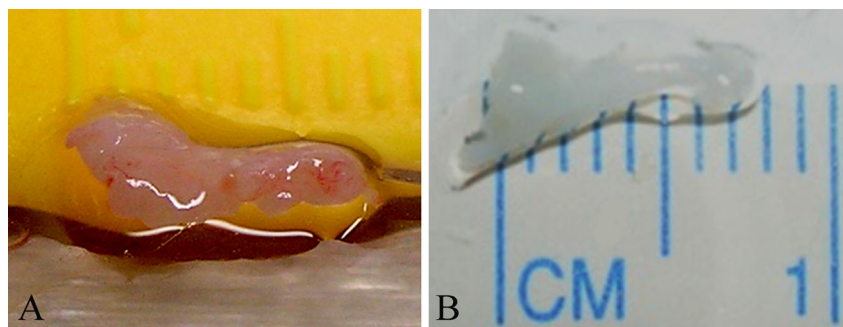
We then decided to process the tissue with 1-h water step and to reduce the timing of deoxycholate treatment to 1 h. After one cycle of the modified decellularization process, bile ducts macroscopically appeared similar (Fig. 2). The average length of native and treated bile ducts (1.08 ± 0.45 and 0.7 ± 0.1 cm, respectively) was not significantly different ($p > 0.1$), even if a decrease after the decellularization process was revealed. Further analysis of treated bile duct showed that tissues appeared completely decellularized and structurally intact. No cells and nuclear material were identifiable by H&E (Fig. 3a, b). As shown in Fig. 3a, native bile ducts are characterized by epithelial cells limiting the body lumen, and by a smooth muscle cell layer. After decellularization, an acellular matrix was observed (Fig. 3b) with an undamaged and sharply defined fiber structure. The epithelial cells of the inner lining and mucous glands were completely absent (Fig. 3b). No nuclear material was detected by DAPI staining (Fig. 3c, d). Moreover, Movat pentachromic staining showed an undamaged net of collagen and elastic fibers, suggesting that the three-dimensional architecture and the protein network of the decellularized bile ducts remained intact with little structural perturbation (Fig. 3e, f). Furthermore, cell nuclei, myocytes as well as amorphous substance, present in the native bile ducts (Fig. 3e), were totally absent in the decellularized matrix.

SEM analysis showed that acellular bile duct matrix was characterized by intact collagen fibers both on the external and the internal surface (Fig. 4a–d). Additionally, no difference in the quantity of elastin (per wet weight) present in the decellularized samples versus native tissues was seen (Fig. 4e). Taken together, the results suggested that one cycle of the modified decellularization protocol is sufficient to remove cellular components while preserving the network of collagen and elastin fibers which represent the main components of the bile duct structural matrix.

4 Discussion

The bile required for food digestion is carried from the liver to the small intestine by the bile ducts. Blockage of the bile duct

Fig. 2 Macroscopic view of native (a) and decellularized (b) bile ducts



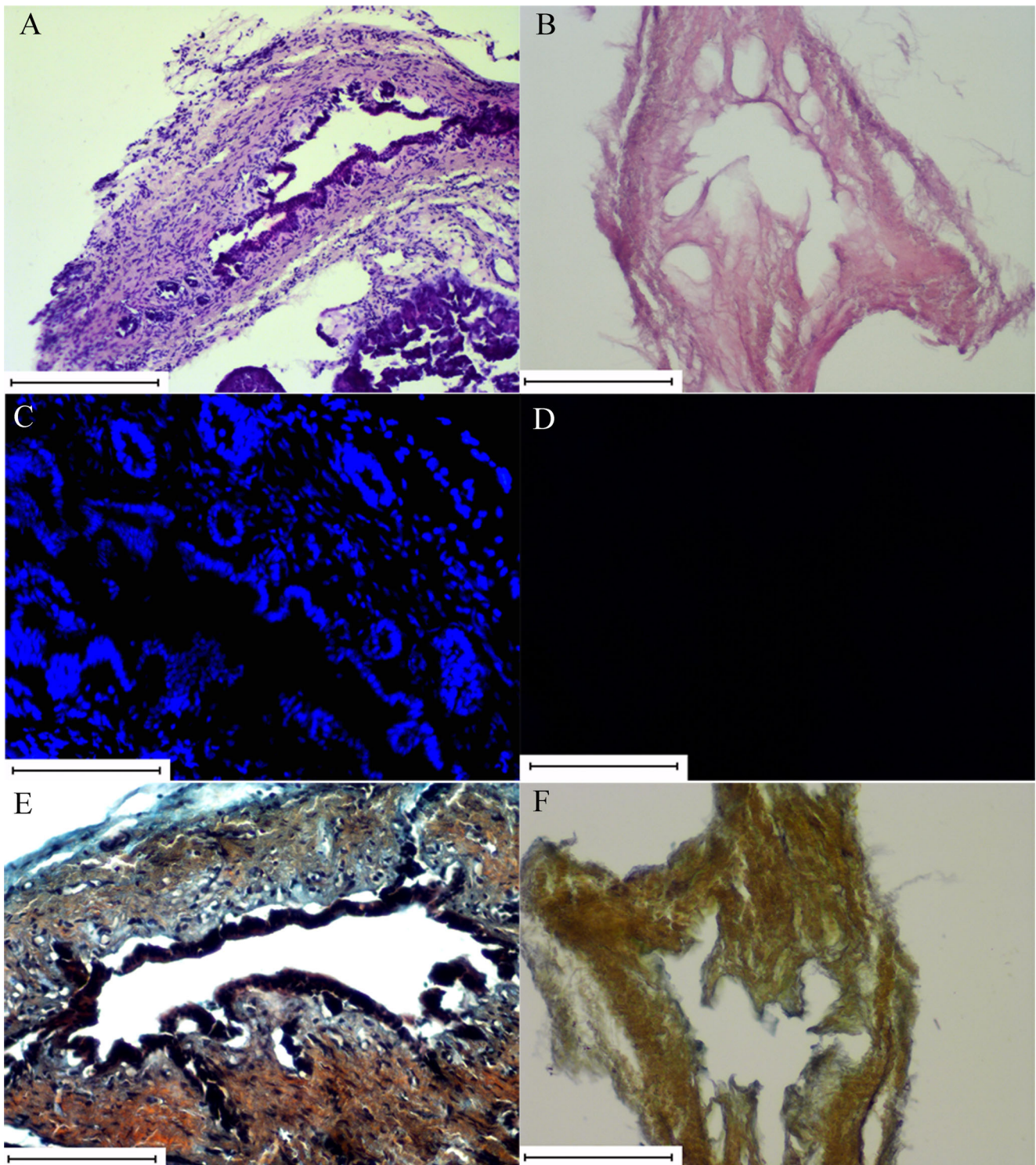


Fig. 3 Hematoxylin and eosin (a, b), DNA (c, d), and Movat pentachromic (e, f) staining of native (a, c, e) and decellularized (b, d, f) bile ducts. *Black* indicates nuclei and elastic fibers, *red* smooth muscle, while *yellow* collagen and reticulum fiber (a: scale bar = 50 μm ; b-f: scale bar = 100 μm)

by congenital (atresia), inflammatory, or acquired diseases prevents the bile from being transported to the intestine, leading to life-threatening infection, sepsis, and chronic liver disease. Bile duct injuries are currently treated using endoscopic techniques (such as bile duct stents) and/or reconstructive

surgical procedures [12, 13] which, however, are often associated with severe side effects. Moreover, in the presence of extensive lesions, the only possible surgical solution is a hepatico-jejunostomy (anastomosis of the hepatic duct to the jejunum) [12]. Disturbances in the release of gastrointestinal

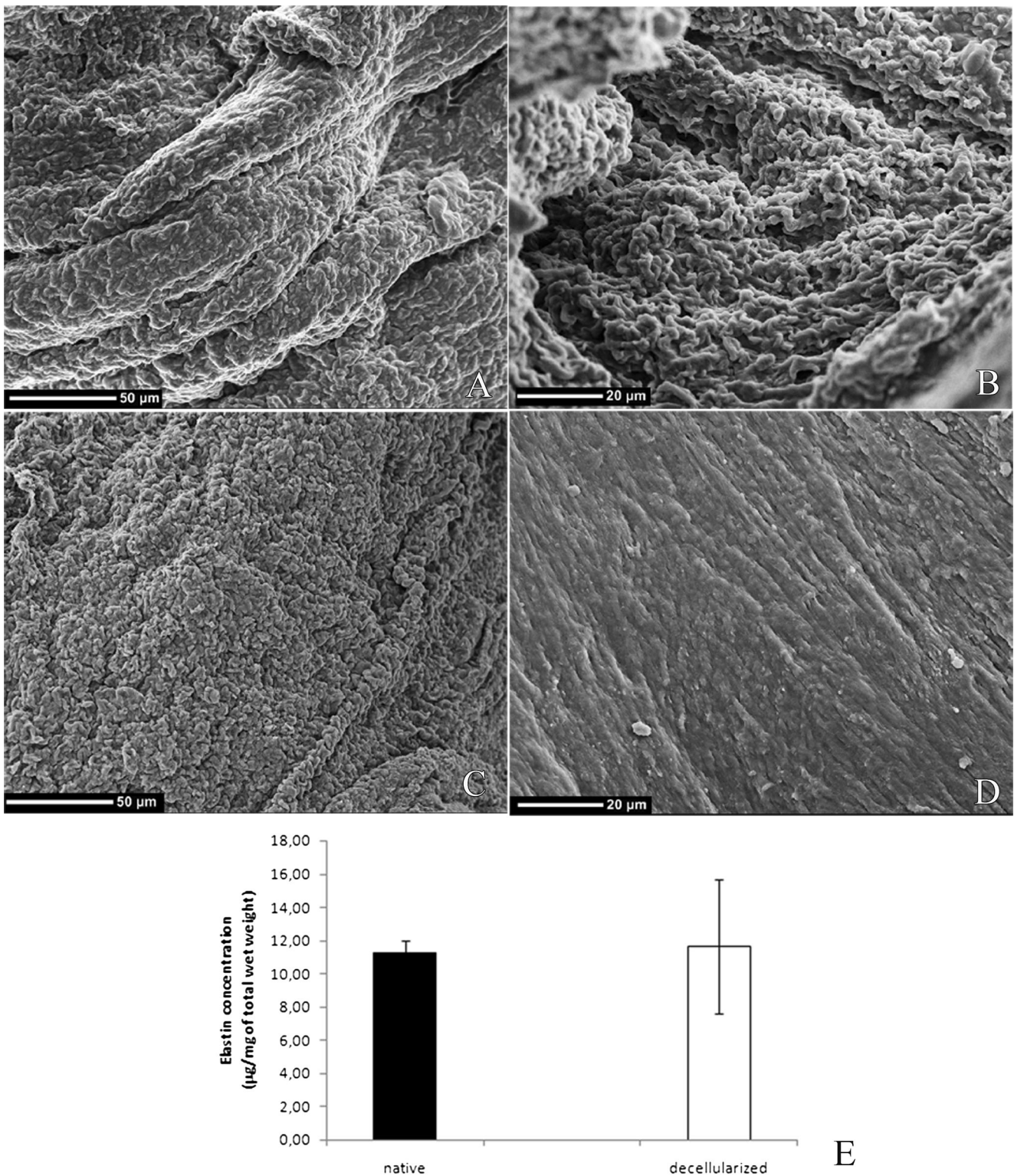


Fig. 4 SEM micrographs of external (a, c) and internal (b, d) part of native (a, b) and decellularized (c, d) bile ducts. e Elastin contents (μg/mg of total wet weight) of native and decellularized bile duct samples (a, c: scale bar = 50 μm; b, d: scale bar = 20 μm)

hormones, altered bile synthesis and gastrin releasing, with consequent duodenal ulcer formation have been frequently observed after this approach [12, 14]. The availability of grafts

similar to the native duct tissue, allowing the bile to drain normally, without leaving traces of foreign matter in the body, could have therapeutic potentials for patient treatment.

Different bile duct grafts, made of biological (i.e., collagen) or synthetic materials, have been evaluated in in vivo preclinical studies and supported the feasibility of a tissue engineering therapeutic option for patients with bile duct injury and stenosis [15].

Based on the idea that the natural extracellular matrix (ECM) can be considered for the development of tissue-engineered scaffolds [16], herein, we evaluated the efficacy of the previously developed DEM approach to obtain rat bile duct acellular matrices [5–9]. The goal of decellularizing an allograft is to remove virtually all of the immunogenic cellular components, while preserving the structural, biochemical, and mechanical property of the tissue. Results demonstrated the almost complete absence of cells and cell membranes and confirmed the decellularization of the bile duct matrix. It is well known that the structure and the composition of ECM influence cell adhesion and differentiation and provide critical physical cues that orchestrate tissue formation and function [17, 18]. Our results showed that DEM did not significantly alter the morphology and tissue architecture of the bile duct matrix, demonstrating that the ECM three-dimensional structure was well preserved. Moreover, matrix content evaluation revealed that the decellularized bile duct matrix contained the same concentration of ECM components that are found in the native bile ducts, suggesting that the DEM process was able to retain elastin, which have been shown to have an effect on cell behavior [19].

5 Conclusions

The DEM procedure results in decellularized bile duct matrices with good preservation of most of the ECM structural components. This one-cycle approach is quick and effectively results in a bile duct scaffold that retains an adequate microstructure that should prove suitable for tissue engineering applications. Considering the small dimensions of rat bile ducts, further studies in larger animals (such as pig or non-humans primates) will be necessary to fully characterize the obtained decellularized matrix and to develop a suitable bioengineered bile duct graft. Moreover, once obtained, its biocompatibility will be evaluated by in vivo implanting both decellularized and re-cellularized scaffolds. In particular, the development of a columnar epithelium and of a proper innervations will be carefully considered.

Acknowledgments This work was supported by a grant (pd 239-28/04/2009, GRT 1210/08) issued on 28 December 2008 by the region Tuscany (Italy) entitled “Clinical laboratory for complex thoracic respiratory and vascular diseases and alternatives to pulmonary transplantation.”

Compliance with ethical standards The publication of this manuscript was approved by the Institutional Review Board of the Kazan Federal University. The study was approved by the Animal Care and Use Committee and the Bioethics Committee of the University of Florence (Italy).

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