



Full length article

Heparin-gelatin mixture improves vascular reconstruction efficiency and hepatic function in bioengineered livers



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ABSTRACT

Whole organ decellularization is a cell removal process that creates a natural extracellular matrix for use in transplantation. A lack of an intact endothelial layer in the vascular network of decellularized organs results in blood clotting even with anti-coagulation treatment. Furthermore, shear stress caused by blood flow may affect reseeded parenchymal cells. We hypothesized that a heparin-gelatin mixture (HG) can act as an antithrombotic coating reagent and induce attachment and migration of endothelial cells (ECs) on vascular wall surfaces within decellularized livers, with subsequent parenchymal cell function enhancement. Portal vein (PV) perfusion was performed for right lateral lobe decellularization of porcine livers. We tested if HG-precoating of isolated decellularized PV could increase EC attachment and migration. Additionally, we coated PV and hepatic artery walls in decellularized liver with HG, and then repopulated it with ECs and maintained it under vascular flow in a bioreactor for 10 days. Re-endothelialized scaffolds were perfused with porcine blood for thrombogenicity evaluation. We then co-cultured hepatocellular carcinoma (HepG2) cells and ECs to evaluate the effect of endothelialization on parenchymal cells. Finally, we transplanted these scaffolds heterotopically in pigs.

HG improved ECs' ability to migrate and adhere to vessel discs. ECs efficiently covered the vascular compartments within decellularized scaffolds and maintained function and proliferation after HG-precoating. No thrombosis was observed after 24 h blood perfusion in HG-precoated scaffolds, indicating an efficiently endothelialized vascular tree. HepG2 cells displayed a higher function in scaffolds endothelialized after HG-precoating compared to uncoated scaffolds *in vitro* and after *in vivo* transplantation. Our results lay the groundwork for engineering human-sized whole-liver scaffolds for clinical applications.

Statement of Significance

A major obstacle to successful organ bioengineering is vasculature reconstruction to avoid thrombosis and deliver nutrients through blood to the whole scaffold after *in vivo* transplantation. Although many attempts have been made to construct endothelial cell layers on the vascular network within decellularized organs, complete coverage has not been achieved. Here, we describe an effective approach for endothelial cell seeding to reconstruct a patent vascular tree within decellularized livers by coating the vasculature using heparin-gelatin mixture. Our results have demonstrated that enhancement of endothelial cell attachment by heparin-gelatin treatment could improve vascular patency and parenchymal cell function *in vitro* and *in vivo*. These results represent a significant advancement toward bioengineering functional liver tissue that maintains vascular patency for transplantation.

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1. Introduction

End-stage liver disease (ESLD) is a global problem. Liver transplantation is currently the only option for treatment of ESLD, however the availability of this option to patients is limited by

profound shortage of transplantable organs [1]. According to a report by the Organ Procurement and Transplantation Network (2012), more than 17,000 people in the United States were on a waiting list for a liver transplant. Approximately 40% of listed patients each year do not receive a liver transplant, and a significant number of these patients either die or become too sick for transplantation [2]. To overcome this problem, scientists are investigating alternative therapies through tissue engineering. The decellularization technique was designed to remove cells and debris from tissues and organs while preserving biochemical composition, biological activity, three-dimensional organization and integrity of the extracellular matrix, indicating the maintenance of native ECM-contained cues necessary for the reseeded cells [3–5]. It is important that engineered solid organs such as the liver, kidney, and heart should be structurally and functionally similar to the native state [6–8]. Decellularized livers lack an endothelial cell lining, subsequently thrombogenicity is a common complication during extracorporeal blood perfusion or after *in vivo* transplantation [9–11]. Lining of blood vessels by ECs is essential to provide a non-thrombotic barrier within decellularized organs and ensure that blood flow *in vivo* is restricted to the vascular spaces and protect parenchymal cells from shear stress [12]. To address this issue, we have developed a simple and safe technique to allow efficient re-endothelialization of the decellularized right lateral lobe of porcine liver.

Blood vessels in decellularized livers express important growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which attract endothelial cells upon recellularization, however absence of the natural endothelial layer may result in cells migrating into the parenchyma rather than attaching to blood vessels walls [13]. We hypothesized that by coating the blood vessels with gelatin, the adhesion of reseeded endothelial cells on the vascular surface would be improved, and migration into parenchyma would be decreased. Moreover, to increase the ability of vessel surfaces to induce cell attachment and spreading, cell-adhesive molecules in the vessel surface can be used [14]. Heparin is a good option that can be applied since it has a non-thrombogenic characteristics and its ability to bind with endothelial cells [15,16]. Because of its highly charged nature, heparin interacts in a non-specific manner with a variety of plasma proteins including fibronectin, vitronectin, and platelet-derived growth factor [17–19]. Molecular weight and charge density are important factors that determine the efficiency of heparin binding to endothelial cells [18,20]. The higher charge density and high molecular weight of heparin have been shown to enhance the degree of binding to endothelial cells [16]. Our aim was to coat blood vasculature surfaces in decellularized right lateral lobes of porcine livers using a heparin-gelatin mixture, followed by infusion of endothelial cells to bioengineer a fully endothelialized human-sized organ.

2. Materials and methods

2.1. Organ collection and decellularization

All animal procedures were conducted in compliance with the guidelines approved by the Institutional Animal Care and Use Committee (Kangwon National University, South Korea). Porcine livers were collected from adult pigs (40–50 kg) immediately after desanguination through the cervical vessels. Decellularization of the right lateral lobe was performed as described in [Supplementary material S1](#).

2.2. Histological analysis

Samples of native and decellularized livers were fixed in 10% neutral buffered formalin and then embedded in paraffin.

Four-micron-thick sections were cut, deparaffinized, and then stained with hematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) stain. To confirm the efficiency of our technique for removing nuclear and cytoplasmic materials, DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St Louis, MO, USA) was used to evaluate the presence of nuclear material. For scanning electron microscopy (SEM), samples were fixed in cold 2.5% glutaraldehyde in phosphate buffered saline (PBS), then dehydrated in ascending dilutions of alcohol before critical point drying with carbon dioxide. Samples were sputter coated and visualized with a scanning electron microscope (Carl Zeiss, Jena, Germany).

2.3. DNA quantification

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions then quantified using the NanoDrop2000c (PiqLab, Erlangen, Germany).

2.4. Western blot analysis

Total protein content was determined using (Bio-Rad Laboratories, Hercules, CA). Key liver ECM proteins including laminin, fibronectin, and collagen as well as (1,3)-galactose (α -gal) were analyzed using Western blot (see [Supplementary material S2](#)).

2.5. Growth factor assays

Native and decellularized portal veins were lyophilized and suspended in urea-heparin extraction buffer. The extraction buffer consisted of 2 M urea and 5 mg/ml heparin in 50 mM Tris with protease inhibitors at pH 7.4. The extraction mixture was shaken at 4 °C for 30 h and then centrifuged at 13,000g for 30 min. Supernatants were collected and the concentration of total protein in each supernatant was measured by the Bradford protein assay according to the manufacturer's protocol. VEGF and bFGF were quantified using pig VEGF (Biotain Pharma Co., Ltd., China) and human bFGF (Sigma-Aldrich) enzyme linked immunosorbent assay (ELISA) kits.

2.6. Culture of human EA.hy926 endothelial cell line and hepatic carcinoma cells (HepG2 cells)

Human EA.hy926 endothelial cells/HepG2 hepatic carcinoma cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% P/S in a humidified incubator at 37 °C and 5% CO₂. Suspensions of EA.hy926 cells/HepG2 were obtained from confluent cultures using trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA-4Na; Gibco/BRL) followed by determining the cell concentration using a hemocytometer.

2.7. *In vitro* cell attachment assay

To evaluate the ability of the blood vessels to support the attachment of EA.hy926 ECs, portal veins were isolated after decellularization and sterilization of the right lateral lobe of porcine livers and discs of 9–10 mm in diameter were cut and placed in a 48-well plate. The discs were treated with either 0.1% gelatin (Sigma-Aldrich) containing 0.1 wt% heparin sodium salt (H4784; Sigma-Aldrich), 0.1% heparin or 0.1% gelatin for 1 h. Discs of vessels treated by PBS were used as a negative control. After 1-h treatment, the solutions were aspirated, then 300 μ L of cell suspension containing 20 \times 10³ EA.hy926 ECs were applied to the disc surfaces. The plate

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