31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Whole Organ Decellularization - A Tool for Bioscaffold Fabrication and Organ Bioengineering

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Abstract— **The use of synthetic and naturally-derived scaffolds for bioengineering of solid organs has been limited due to a lack of an integrated vascular network. Here, we describe fabrication of a bioscaffold system with intact vascular tree. Animal-donor organs and tissues, ranging in size up-to thirty centimeters, were perfused with decellularization solution to selectively remove the cellular component of the tissue and leave an intact extracellular matrix and vascular network. The vascular tree demonstrated sequential fluid flow through a central inlet vessel that branched into an extensive capillary bed and coalesced back into a single outlet vessel. In one example, the liver, we used central inlet vessels to perfuse human and animal liver cells through the bioscaffold to create a functional liver tissue construct *in vitro*. These results demonstrate a novel yet simple and scalable method to obtain whole organ vascularized bioscaffolds with potential for liver, kidney, pancreas, intestine and other organs' bioengineering. These bioscaffolds can further provide a tool to study cells in their natural three-dimensional environment, which is superior for drug discovery platform compared with cells cultured in two-dimensional petri dishes.**

I. Introduction

Solid organ transplantation is a victim of its own success. As results have dramatically improved, the demand for transplantable grafts has increased but the offer has not kept pace [1]. Therefore, the gap between the number of patients who have received a transplant and those who are in the waiting list has become wider than ever; also, the mortality while on the waiting list is increasing. Over the years, alternative sources of organs were investigated, including: xenotransplantation and tissue engineering [TE]. Xenotransplantation is the transplantation of living cells, tissues or organs from one species to another; thus far, organ transplant from animals to humans has been impossible because of the overwhelming rejection and the risk of transmitting animal viral diseases to humans.

TE uses a combination of cells, biomaterials and suitable biochemical and physio-chemical factors. The goals for TE are to replace damaged and non-functioning tissues or organs with constructs obtained through the seeding of functional cells within a structure capable of: 1) supporting the three-dimensional [3D] tissue formation; and 2) mimicking the function of the natural extracellular matrix [ECM]. Such structure is referred to as scaffold. However, the main roadblock towards the production of viable constructs is the inadequacy of the current technology to reproduce: i) those signals through which cells interact with one another and with the ECM, and ii) the vasculature within the scaffold, which is essential for oxygen and nutrient supply to cells.

The ECM provides structural support to cells, segregates tissues from one another, and regulates intercellular communication and cell's dynamic behavior. Importantly, the ECM contains several bioactive molecules which, in their unique spatial distribution, provide a reservoir of biologic signals that are difficult to artificially replicate. Notably, although these biomolecules are present within ECM in very small quantities, they act as potent modulators of cell behavior.

We employed tissue decellularization to obtain collagen-rich bio-scaffolds for tissue engineering of urological tissues such as the bladder and urethra [2, 3]. However, these were relatively "thin" tissues. In order to decellularize bulky tissues such as pig liver the tissue was sectioned into thin slices which could be completely decellularized to obtain a 2D ECM preparation [4] The biomatrix was seeded with hepatocytes that preserved their function for several weeks. This data provided the evidence that liver biomatrix may be a superior alternative to existing scaffolds for tissue engineering for the following reasons: the liver ECM is bioresorbable, it can be easily prepared, and it supports long-term hepatocellular functions in vitro. These initial studies were recently followed by researchers from the University of Minnesota that have successfully produced a beating heart through seeding neonatal cardiac cells within the scaffold of a rat heart [5].

In the current study, we applied the principles of whole organ decellularization, while preserving the ECM and the vascular network, as an approach to potentially bioengineer organs such as liver, pancreas, kidney and intestine for transplantation and drug discovery.

II. METHODS AND RESULTS

A. Whole Organ Decellularization

I In order to decellularize large volume tissues without destructing the tissue by sectioning it to thin slices, we used the native vascular system to perfuse a decellularization solution (made of 1% of detergent Triton X-100 and 0.1% ammonium hydroxide in deionized water) throughout the

Manuscript received April 7, 2009. This work was supported in part by a predoctoral fellowship from Fundação para Ciência e Tecnologia, Portugal - SFRH/BD/11802/2003 (PMB); and by the Marie Curie International Outgoing Fellowship POIF-GA-2008-221850 (GO).

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organs by cannulating their largest vessels for vascular access. Perfusion of the decellularization solution is preceded and followed with perfusion of deionized water, a process that takes approximately 2 days. Using this method, we were able to successfully decellularize livers, kidneys, pancreas and intestines of various sizes and animal species. These include an eight centimeter ferret liver (Fig. 1B), a twelve centimeter pig kidney (Fig. 1F), a twenty-three centimeters pig pancreas (Fig. 1J) and a twenty-five centimeters pig small intestine (Fig. 1N). This method produced completely decellularized tissues that demonstrated a preserved vascular network, which we term as an acellular vascularized bioscaffold (AVB). In the particular case of the liver, the vascular tree could be directly visualized due to the transparent parenchymal space (Fig. 1B). The use of a mild detergent Triton X-100 and ammonium hydroxide enables the quick and consistent removal of all the cellular components of the tissue, leaving behind mostly intact the ECM elements, whilst maintaining increased protein complexes in comparison to alternative detergents including SDS [sodium dodecyl (lauryl) sulfate]. Complete decellularization of AVB and the preservation of the vascular network were confirmed with several methods. H&E staining of paraffin sections of the numerous organs decellularized (Fig. 1C, G, K, O) showed the expected pink eosinophilic staining for collagen with no basophilic staining of cellular material. Further analysis of these decellularized bioscaffolds using antibodies for several types of collagen, laminin and fibronectin showed preservation of the distinctive matrix chemistry of each organ and maintenance of their spatial locations (data not shown). To confirm the integrity of the vascular network we tested if fluid injected into the vasculature can flow through the vasculature and not extravasate throughout the organ. An x-ray fluoroscopic study with radio-opaque dye demonstrated that the injected dye was flowing as expected from an intact vascular network and slowly moved from larger vessels to smaller capillaries (Fig. 1D, 1H, 1L and 1P). Approximately 5 minutes after perfusion started, the whole organs became radio-opaque, suggesting some leakage of the dye from the vascular channels into the matrix. Nonetheless, this series of experiments demonstrated that the AVB prepared from different organs maintained patency of their original vascular network. Such intact network can be used to deliver cells into the bioscaffold and subsequently to perfuse nutrients to the bioengineered organ.

B. Bioscaffold Recellularization

Besides providing vascular channels, the bioscaffolds can also provide adequate environment for cell growth. The liver is composed of two major cell types; hepatocytes and endothelial cells. To investigate if endothelial cell seeding was possible, GFP-labeled mouse endothelial cells were infused through the portal vein and the liver AVB was perfused with endothelial cell media for 3 days. The seeded AVB was visualized under fluorescent microscope, showing a single line of fluorescent endothelial cells lining the vascular channels (Fig 2A). No labeled endothelial cells were observed outside the channels. To test if we can also recellularize the portal vein vascular tract, we seeded the AVB with the same fluorescently-labeled endothelial cells (green) through the portal vein and inspected the AVB under fluorescent microscope. The peri-portal space was filled with endothelial cells (Fig 2B), showing a characteristic hexagonal arrangement of the vascular structures of the peri-portal spaces of the lobules. A picture of one of the lobes showed homogenous distribution of endothelial cells throughout the whole liver AVB (Fig. 2C).

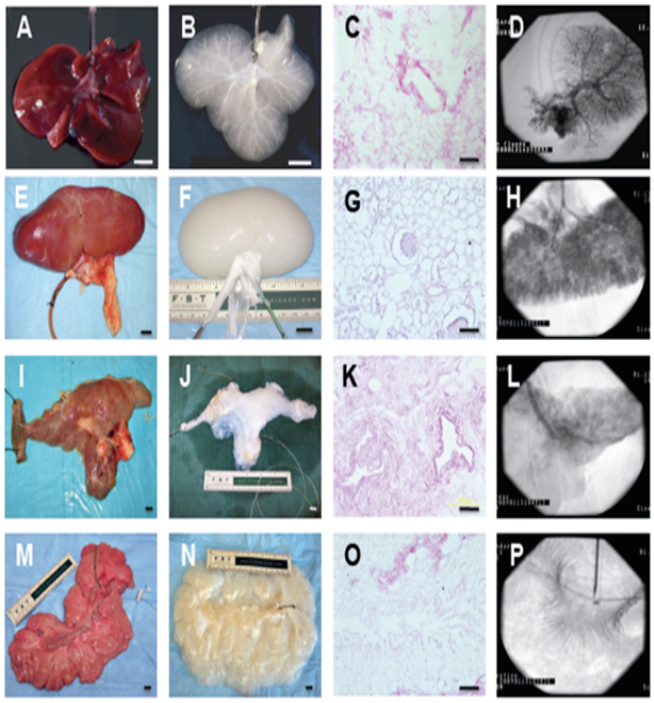

Fig. 1. Organ Decellularization. Organs were obtained en-bloc including arterial and venous structures. Vessels were cannulated and attached to a peristaltic pump, followed by overnight perfusion of ddH2O. Organs were then decellularized with 1% Triton-X / 0.1% Ammonium hydroxide in ddH2O solution at 10–60 ml per hour for 24 hrs or until translucent, and perfused with ddH2O prior to sterilization (gamma irradiation) and cell-seeding studies or processed for histological analysis. (A, B; E,F; I,J; M,N) Macroscopic view of fresh ferret liver, pig kidney, pancreas and intestine, before and after decellularization, respectively. The removal of the cellular components is observable with the transparency/ white color of the decellularized bioscaffolds. (C, G, K, O) H&E staining of histological sections of the decellularized liver, kidney, pancreas and intestine, respectively. No nuclear cellular material is observable and only pink eosinophilic staining expected from proteinous extracellular matrix is apparent. (D, H, L, P) Fluoroscopic analysis of the vascular network of the decellularized liver, kidney, pancreas and intestine, respectively. Contrast agent flows through the decellularized organ scaffolds demonstrating progressive flow from large vessels branching into medium-sized arterioles and continues to ultimately fill the fine vasculature of each organ. Scale bars are 1cm in Fig. 1A-B, E-F, I-J and M-N and 100um in Fig. 1C, G, K and O.

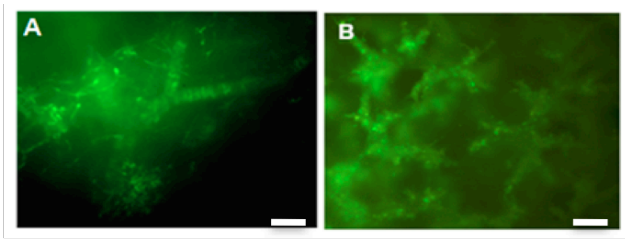

Fig. 2. Re-endothelialization of the ferret liver bioscaffold. (A) GFP-labeled EC were seeded via *vena cava* in the liver bioscaffold, demonstrating endothelial cells that are lining the bioscaffold's vascular channels. (B) GFP-labeled EC perfused via the portal vein distribute predominantly in the hexagonal shape peri-portal areas. Scale bars 100um.

Taken together, these results suggest that the perfusion method used to deliver the decellularization solution can be used to deliver cells for seeding the lumen of the vascular channels and the parenchyma of the liver lobules, by using the portal vein and the vena cava, respectively. It also confirms that the vascular channels are intact and that mostly no endothelial cells can be observed outside the vascular channels.

We further performed a series of co-seeding experiments of endothelial cells and human hepatocellular carcinoma, HepG2, cells. 30 million HepG2 and 30 million endothelial MS1 cells were seeded through portal vein of the bioscaffold by perfusion with culture medium. Culture medium (DMEM w/ 10% FBS) was then continuously perfused for 1 week at 6 ml/min. One week after seeding, high density of cells can be observed throughout the AVB with visible tissue formation (Fig. 3A). Immunohistochemical analysis showed extensive and intense albumin expression (Fig. 3B) and a large number of proliferating cells in the core of the bioscaffold, as evident by Ki67 immunostaining (Fig 3D). Von Willebrand Factor staining showed a pattern typical of a cross section through capillaries (Fig. 3C). Together, these experiments showed the potential of bioscaffold recellularization using cell perfusion as an efficient approach for the bioengineering of whole organs.

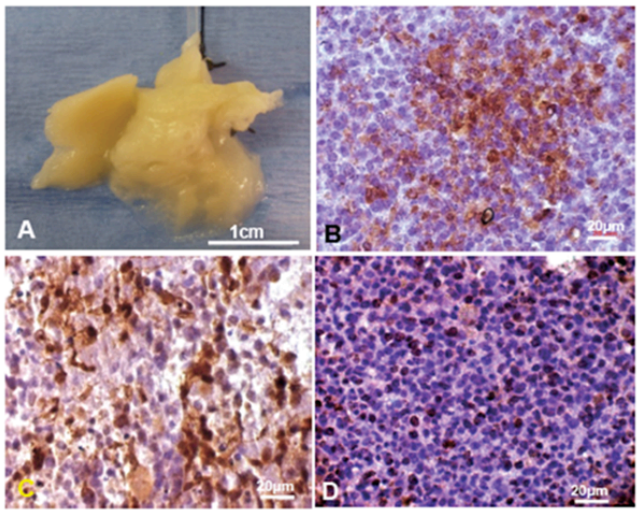

Fig. 3. Re-cellularization of the ferret liver bioscaffold. (A) Macroscopic appearance of a right lobe of a ferret liver bioscaffold seeded with human hepatocyte progenitors (HepG2) and endothelial cells. (B) Immunostaining for albumin expression of HepG2 cells engrafted in the bioscaffold. (C) Von Willebrand Factor expression by seeded endothelial cells with a pattern similar to capillary networks. (D) Anti-Ki67 immunostaining shows a large number of proliferating cells within the bioscaffold.

III. Discussion

The current study describes an effective method to fabricate organ bioscaffolds with a complete vascular system. Our data showed that the microarchitecture and the vascular network of different organs processed in this method were maintained intact and supports cell growth in vitro. This approach, which produces intact whole organ bioscaffolds with the preservation of the native microarchitecture, can provide a material with unique properties for whole organ bioengineering. The three dimensional vascularized structure can support the growth and viability of different cell types, facilitating a new level of complexity for cellular interaction, organization and perfusion; an unmet need in regenerative medicine.

Decellularization of tissues has traditionally been performed by agitating the tissue of interest in a container and allowing the cells to decellularize in bulk from an outside in approach [6]. Such an approach has been effective in completely decellularizing tissues only up to five millimeters in thickness [7]. Thicker tissues tend to decellularize well at the surface, but the core remains cellular. The reason is because the decellularized matrix at the surface forms a resistant layer preventing efficient access of the detergent to the deeper parenchyma. The method described here is less traumatic to the tissue than the classic agitation approach and thus results in an acellular matrix that demonstrates a patent vascular tree that can be used to deliver cells and nutrients into the bioscaffold. The choice of detergent for the generation of AVB by perfusion may influence the preservation of important biological activities. Harald C Ott et al recently reported the use of this technique in heart decellularization with similar results, confirming the potential of this novel method to generate scaffolds for bioartificial organ engineering [5]. Although the use of strong ionic detergents such as SDS facilitates complete removal of cells and can yield a functional bioscaffold, it is possible that it may damage some ECM components [8]. Therefore, we opted to use a mild non-ionic detergent, Triton X-100. We found that this detergent could successfully decellularize the whole liver, kidney, lungs and small intestine by the removal of approximately 98% of cellular DNA. In fact, the ability of perfused endothelial cells and hepatocytes to localize specifically to appropriate sites within the liver bioscaffolds suggests that normal physiological cues for homing and function were successfully preserved.

ECM derived from organs such as the small intestine [9], urinary bladder [2] or skin [10] are now widely used for the reconstruction of many different tissues. Lower urinary tract reconstruction, arterial graft, or skin reconstitution are amongst the numerous clinical applications. In the specific case of the liver, decellularized liver matrix sections have been used for liver tissue engineering [4]. However, these acellular tissue ECMs do not possess a natural vascular tree that is essential to support the bioengineering of a three-dimensional and bulky bioartificial organs. These results suggest that the AVB has an important advantage over other ECM preparation methods that do not preserve an intact vascular network. It makes possible to overcome the oxygen

and diffusion limitations imposed by tissue thickness. This is also true for organs like the pancreas and kidney, where so far no record has been found in the literature of a successful method to prepare a whole organ scaffold that could successfully enable kidney or pancreas bioengineering.

Finally, the major implication of this work is the simple method of generating a biodegradable, biocompatible, vascularized organ bioscaffold with the equal amount of complexity as that seen in nature. These bioscaffolds may be used for the bioengineering of other solid organs that require a vascular tree to support a large number of cells, generating new drug discovery platforms and most needed organs for transplantation.

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